The Dissertation Committee for Taegyu Kim Certifies that this is the approved version of the following dissertation:

Rainwater Harvesting: The Impact of Residential-scale Treatment and Physicochemical Conditions in the Cistern on Microbiological Water Quality

Committee:

Mary Jo Kirisits, Supervisor

Kerry A. Kinney

Dennis J. Lye

Howard M. Liljestrand

Dean R. Appling

Rainwater Harvesting: The Impact of Residential-scale Treatment and Physicochemical Conditions in the Cistern on Microbiological Water Quality

by

Taegyu Kim, B.S., M.S.

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Dedication

For Ian, Aiden, and Seonju.

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Rainwater Harvesting: The Impact of Residential-scale Treatment and Physicochemical Conditions in the Cistern on Microbiological Water Quality

Taegyu Kim, Ph. D.

The University of Texas at Austin, 2017

Supervisor: Mary Jo Kirisits

Rainwater harvesting (RWH) at an individual residence is an alternative method of water supply for potable and non-potable uses. However, raw harvested rainwater and household-treated rainwater frequently contain a substantial number of unidentified microorganisms, some of which might be human pathogens. The objectives of this study were to understand the microbiological quality of harvested rainwater at residential RWH systems and to understand temporal changes in the rainwater cistern microbiome. To achieve these objectives, physicochemical/microbiological water quality parameters and the harvested rainwater microbiome were analyzed at the cistern and finished cold-water taps of residential RWH systems over the period of one year. Additionally, the impact of physicochemical conditions in the cistern on microbiological water quality was studied in bench-scale cisterns over a 28-day period. In the residential RWH systems, potential human pathogens (*Mycobacterium avium*, *Mycobacterium intracellulare*, *Aspergillus flavus*, *Aspergillus fumigatus*, and *Aspergillus niger*) were found frequently in cisterns and in treated rainwater delivered at the tap; *Legionella pneumophila* was not detected as frequently, but it persisted in a system after its first detection. The dissolved organic carbon (DOC) concentration was positively rank-correlated with heterotrophic plate counts (HPC)

and *L. pneumophila* in non-chlorinated cisterns. The harvested rainwater microbiome was diverse and distinct between RWH sites. The diversity of the rainwater microbiome was correlated with HPC and DOC concentrations. The non-chlorinated cisterns had very stable microbiomes over the period of a year, suggesting that fresh rainfall does not change the cistern microbiome substantially. Filtration/ultraviolet-treatment changed the composition of the harvested rainwater microbiome, but DNA from two genera that contain potential human pathogens (*Mycobacterium* and *Legionella*) still were found in most samples. The bench-scale cistern experiments showed that the cistern microbiome proceeded towards its pre-disturbance state after an influx of fresh roof-harvested rainwater. The *L. pneumophila* concentration decreased over time in all the cisterns, even though HPC in the cisterns were stable over 28 days. Chlorination effectively inactivated *L. pneumophila* in the cistern but only temporarily impacted HPC and the relative abundance of operational taxonomic units in the Firmicutes (e.g., *Clostridium* spp.).

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1. INTRODUCTION

1.1. BACKGROUND

Traditional water supplies are decreasing, while demand is increasing. For example, as shown in Figure 1.1, 99% of the state of Texas suffered from severe drought in 2011 (Brewer 2011), while a 45% increase in population is expected from ca. 2010 to 2040. Fresh groundwaters and surface waters are limited resources, so water conservation and alternative water sources are increasingly important topics. As an alternative method of providing water, rainwater harvesting (RWH) at individual homes is emerging, especially in areas lacking access to other freshwater supplies. RWH is considered a form of reuse by the United States Environmental Protection Agency (USEPA), and it also is one of the recommended water management strategies suggested in the 2012 Texas State Water Plan (TWDB 2012).

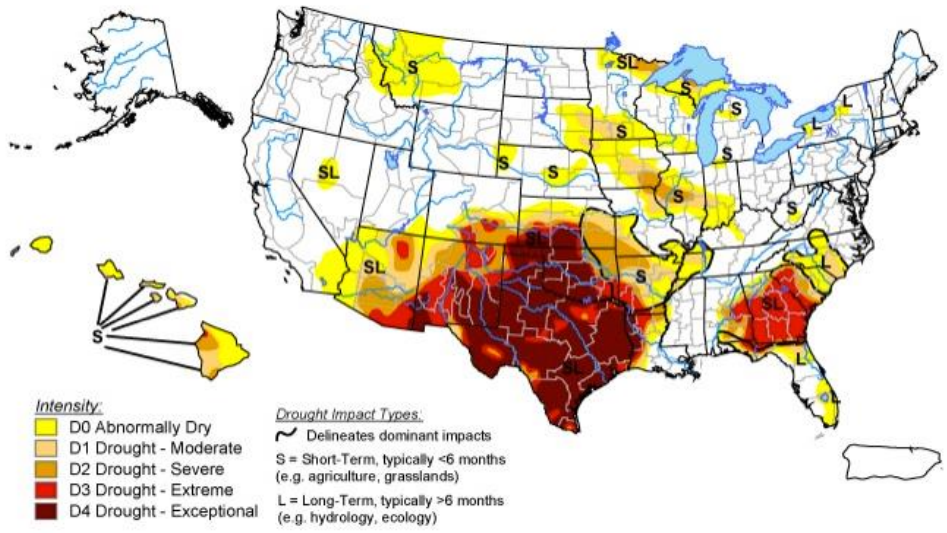

Figure 1.1: U.S. drought monitor in September 2011 (Brewer 2011).

As shown in Figure 1.2, a typical residential RWH system consists of a (1) catchment system (i.e., roof); (2) conveyance system (i.e., piping); (3) pre-treatment system (e.g., first-flush diverter to divert roof runoff at the beginning of a rain event, gutter guards to prevent entry of leaves and other large organic debris into the cistern, roof-wash filter between the roof and cistern to remove large debris); (4) rainwater storage cisterns; (5) pumps; and (6) water treatment units (e.g., filters and ultraviolet [UV] disinfection system) (Yudelson 2010). Harvested rainwater can be used for non-potable (e.g., laundry, showering, toilet-flushing) and potable (e.g., drinking and cooking) purposes.

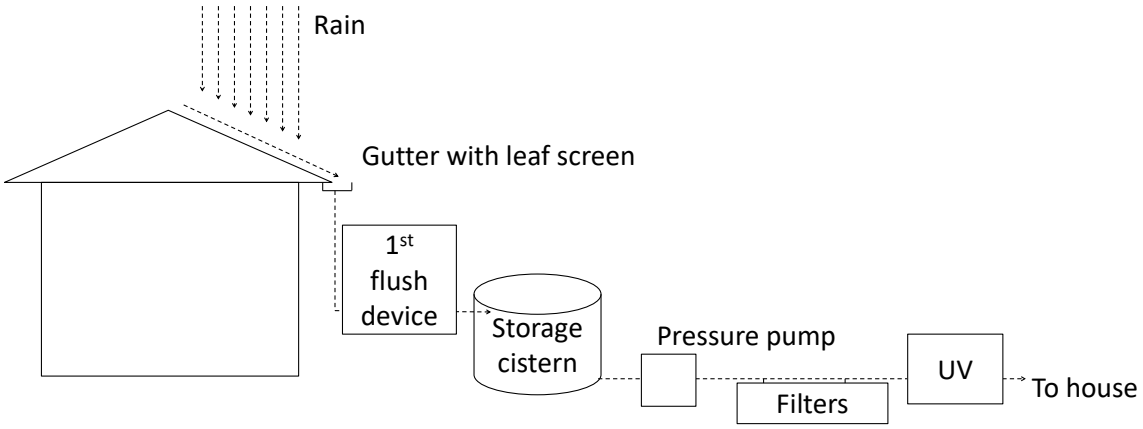

Figure 1.2: Example rainwater harvesting system for potable use.

1.1.1. Benefits of rainwater harvesting

A RWH system is an example of a decentralized water supply system, and, as such, it provides several societal benefits. First, RWH reduces the use of potable mains water and curtails piping costs, which can sometimes be exorbitant. For example, the extension of drinking water distribution lines to reach 135 households in Kenton County, Kentucky

was expected to cost $3.4 million in 2013 (Scalf 2013). Second, a RWH system can decrease the amount energy required for water transport; a study from the Stockholm Environment Institute found that 100 gallons of tap water need 0.81 kWh to be transported 10 miles (Salas et al. 2009), but decentralized RWH systems have very short transport distances. Third, RWH might reduce water loss by leakage; it is estimated that 14-18% of daily water use is wasted through aging pipes in the United States, which accounts for 6 billion gallons per day (Festing et al. 2013). Fourth, RWH can reduce stormwater runoff, thereby improving flood control (Steffen et al. 2013). With these societal benefits, RWH at individual homes is often encouraged as an alternative water supply (Farreny et al. 2011).

1.1.2. Challenges of rainwater harvesting

Harvested rainwater can contain high numbers of bacteria (Lye 1987). Outbreaks due to human pathogenic strains of *Escherichia coli* and *Legionella pneumophila* have been reported from some potable RWH systems (Simmons et al. 2008). RWH systems can be equipped with disinfection systems (e.g., UV light or chlorination) to improve microbiological water quality, but the efficacy of such disinfection systems operated at individual residences is not well-documented. Moreover, despite the existence of potential human pathogens in harvested rainwater, federal water quality regulations do not exist for potable RWH systems at individual residences in the United States. Thus, potable consumers might incur health risks by using harvested rainwater that has not been suitably treated. The research in this dissertation addressed the gaps in our understanding of the microbiological quality of harvested rainwater, which is essential for improving the safety of potable RWH.

1.2. RESEARCH OBJECTIVES AND TASKS

The overarching goals of this research were to understand the quality of harvested rainwater in the cistern, including a careful delineation of the microbiome, and to assess the impact of residential treatment processes on harvested rainwater quality. To accomplish these goals, the specific objectives of the study were as follows: (1) compare rainwater quality before and after treatment in full-scale, residential RWH systems, (2) describe the microbiome of rainwater before and after treatment, (3) assess the impact of physicochemical cistern conditions on the microbiome and on pathogen persistence in rainwater cisterns. The research was accomplished in three tasks, mapping to the three objectives, and a detailed description of each task is given in Chapters 3 - 5.

1.3. DISSERTATION STRUCTURE

1.3.1. Literature review (Chapter 2)

Chapter 2 is a literature review for this dissertation study. It summarizes the previous studies on RWH applications, bacterial quality in the cistern, treatment of harvested rainwater, the microbiome of harvested rainwater, and factors that can change microbiome structure.

1.3.2. Impact of residential treatment processes on harvested rainwater quality (Chapter 3, Task 1)

Problem

Despite the growing popularity of RWH in the United States, harvested rainwater quality at individual homes is not well-understood.

Objective

The objective of this task was to assess water quality in full-scale residential RWH systems and the impact of residential treatment processes on that water quality.

Method

Rainwater samples were collected from six full-scale, residential RWH systems in central Texas, and a suite of water quality parameters (e.g., temperature, turbidity, pH, dissolved oxygen [DO], dissolved organic carbon [DOC], residual chlorine, total trihalomethanes [TTHM], heterotrophic plate counts [HPC], indicator bacteria [total coliform, *E. coli*, and enterococci], and selected opportunistic pathogens) were measured.

1.3.3. Microbiome of harvested rainwater before and after treatment (Chapter 4, Task 2)

Problem

Raw harvested rainwater and treated rainwater contain a substantial number of unidentified heterotrophic bacteria, some of which might be potential human pathogens.

Objective

The objective of this task was to describe the harvested rainwater microbiome in three full-scale residential RWH systems before and after treatment.

Method

DNA was extracted from samples of the cistern and finished-water tap of two RWH systems with filtration/UV-treatment and one RWH system with chlorination/filtration. The 16S rRNA gene was sequenced with MiSeq® Illumina, and the sequences were processed with QIIME (Quantitative Insights Into Microbial Ecology) to analyze the diversity and composition of the microbiome.

1.3.4. Impacts of physicochemical conditions in rainwater cisterns on temporal changes in the cistern microbiome (Chapter 5, Task 3)

Problem

Task 3 is motivated toward understanding how physicochemical factors in the cistern impact temporal changes in the cistern microbiome and the persistence of potential human pathogens such as *L. pneumophila*. In this task, several physicochemical conditions (pH, DOC concentration, presence of sediments, use of batch-chlorination) in the cistern that are at least partly under the control of the rainwater harvester were studied.

Objectives

The objectives of this task were to analyze temporal changes in the cistern microbiome and concentrations of *L. pneumophila* after a disturbance (i.e., an influx of fresh roof-harvested rainwater and *L. pneumophila* to the cistern) and to explore how particular physicochemical conditions affect microbiome recovery and the persistence of *L. pneumophila* in the cistern.

Method

Harvested rainwater was collected from a full-scale RWH system and added to eight bench-scale cisterns, which were adjusted to various physicochemical conditions (pH, DOC concentration, presence of sediments, use of batch-chlorination). The cisterns were perturbed by the addition of fresh harvested rainwater and the introduction of *L. pneumophila*. Temporal changes in the cistern water quality, including the *L. pneumophila* concentration and the microbiome, were monitored over a one-month period.

1.3.5. Task conclusions and future work

Chapter 6 summarizes the conclusions of this dissertation study and presents avenues for future work.

2. LITERATURE REVIEW

2.1. RAINWATER HARVESTING APPLICATIONS

Rainwater harvesting (RWH) refers to the capture and storage of rainwater for future use on-site. RWH is being studied and adopted in countries such as Australia (Ahmed et al. 2012), New Zealand (Simmons et al. 2001), Bangladesh (Karim 2010), South Korea (Lee et al. 2010), Japan (Kobayashi et al. 2014), South Africa (Chidamba & Korsten 2015), Denmark (Albrechtsen 2002), Spain (Farreny et al. 2011), Brazil (Ghisi & Ferreira 2007), Ireland (Li et al. 2010), Greece (Sazakli et al. 2007), and the United States (Lye 1987).

Individual use

Non-treated harvested rainwater quality generally suits outdoor non-potable uses such as gardening (Nolasco 2011), and outdoor use is the most common application of harvested rainwater at individual residences. For example, a survey conducted with 163 American Rainwater Catchment Systems Association (ARCSA) members found that 90% of the individual respondents use harvested rainwater for irrigation (Thomas et al. 2014). In South Korea, roof-top gardening in apartment complexes utilizing harvested rainwater is becoming more popular (Figure 2.1). In these systems, captured rainwater is stored in a roof-top cistern, and residents share harvested rainwater to irrigate their gardens (Jung 2015).

(a) (b)

Figure 2.1: (a) Roof-top gardening (b) harvested rainwater cistern at an apartment complex in South Korea; picture source[1]: (Jung 2015).

From the ARCSA survey, 43% of individual respondents use harvested rainwater for indoor uses (Thomas et al. 2014). Most harvesters treat rainwater prior to non-potable indoor use, such as filtration of rainwater for laundering, toilet-flushing, and showering. Some rainwater harvesters perform a combination of filtration and disinfection (e.g., ultraviolet [UV] light or chlorination) so that they can use harvested rainwater for potable purposes, such as drinking and cooking. The ARCSA survey found that 32% of individual respondents use harvested rainwater for drinking purposes (Thomas et al. 2014).

Commercial building

Because of the economic benefits of RWH, some urban buildings with high demand for non-potable water have installed RWH systems for toilet flushing, irrigation, and cooling purposes. For example, Austin Community College Highland campus, which

[1] Permission to use the pictures granted from the original author.

achieved Leadership in Energy & Environmental Design (LEED) Gold certification, has RWH systems to supply water for toilet flushing (Figure 2.2). It also is common for commercial buildings to capture rainwater and save it for fire hydrant water storage (Park et al. 2006).

(a) (b)

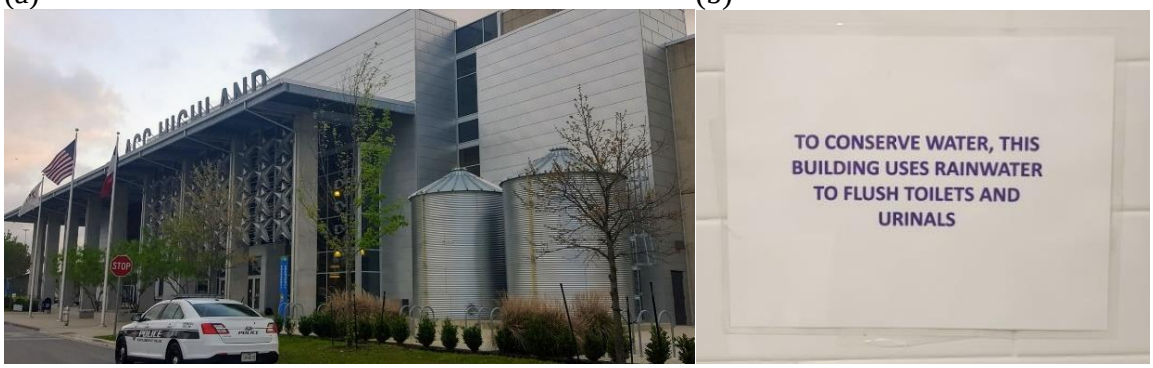

Figure 2.2: Rainwater harvesting cisterns for toilet flushing at Austin Community College Highland campus (a) main building with two rainwater cisterns, (b) public notice at restrooms.

Since RWH is being adopted at residential and commercial buildings, people in these buildings will be exposed to rainwater (via direct consumption or aerosolization). Therefore, it is important to understand the microbiological quality of harvested rainwater.

2.2. MICROORGANISM INTRUSION TO A RWH CISTERN

Fresh roof-harvested rainwater is generally considered to be less polluted than is groundwater. Some studies claim that untreated rainwater is safe to drink (Dillaha III & Zolan 1985), and people drink untreated harvested rainwater in some parts of the world. However, the microbiological quality of harvested rainwater can be impacted in a variety

of ways (Abbasi & Abbasi 2011). First, rainwater collects aerosol bacteria. One study showed that ambient rainwater has high bacterial concentrations, and the concentration of aerosol bacteria drops after a rain event; additionally, aerosol bacterial concentrations are correlated with bacterial concentrations in collected rainwater (Cho & Jang 2014). Second, defecation by birds and other animals on roofs might provide a direct point of entry for potential human pathogens into the RWH system (Ahmed et al. 2011). Third, microorganisms can proliferate during storage in a RWH cistern. For example, (Cho & Jang 2014) captured fresh roof-harvested rainwater and stored it in a pre-sterilized cistern for 13-18 d. They found that bacterial concentrations (by viable plate counts) increased 100-fold, and the adenosine triphosphate (ATP) content increased from 1.9 to 5.0 femtogram (fg) ATP/cell. They also found that the composition of the microbial community changed (i.e., relative abundance of some operational taxonomic units [OTUs] increased while other OTUs disappeared), suggesting that some bacterial groups can survive better than others. In a real RWH system, storage times in the cistern can exceed the 13-18 d tested in the Cho & Jang (2014) study, but how microorganisms react to this long storage time has not been well studied. Given that microorganisms can enter rainwater cisterns and proliferate, careful attention must be paid to the microbiological quality of rainwater intended for potable use.

2.3. INDICATOR BACTERIA AND POTENTIAL HUMAN PATHOGENS IN HARVESTED RAINWATER

Indicator bacteria detection with culture-based techniques

Total coliform (TC), fecal coliform (FC; thermotolerant coliform), enterococci, and *Escherichia coli* are commonly used indicators of bacterial water quality. Recent

monitoring studies of microbial contamination in residential rainwater cisterns found high concentrations of heterotrophic bacteria and fecal coliforms (Ahmed et al. 2010a, Simmons et al. 2001, Lye 1987). For example, substantial HPC (6.5×10^7 colony-forming units [CFU]/mL) were measured in harvested rainwater near the bottom of the storage cisterns (Lye 1987). In the same study, Lye (1987) found high concentrations of TC (average of 600 CFU/100mL) in RWH cisterns. More than 56% of 125 residential RWH systems sampled in New Zealand had measurable FC in 100-mL aliquots of raw harvested rainwater (Simmons et al. 2001). Another study in Australia investigated 100 cistern rainwater samples and found that 58% of the samples were positive for *E. coli* and 83% of the samples were for positive for enterococci (Ahmed et al. 2010a).

Generally, the presence of fecal indicator bacteria suggests fecal contamination of the harvested water, where potential human pathogens might occur. However, Ahmed et al. (2014) showed that the occurrence/concentration of fecal indicators in rainwater was not correlated with occurrence/concentration of potential pathogens in harvested rainwater. Thus, identification and quantification of potential human pathogens in harvested rainwater is an important step for characterizing harvested rainwater quality.

Potential pathogen quantification with DNA-based techniques

Molecular techniques, such as quantitative, real-time polymerase chain reaction (qPCR), have been adopted to analyze DNA signatures from potential human pathogens in various types of waters, including harvested rainwater. One downside of DNA-based techniques is that they cannot distinguish between dead cells and live cells without additional processing steps. It is known that DNA from dead cells can persist for weeks (Nocker et al. 2007); for example, chloroform-sterilized sand showed that DNA from killed

organisms was degraded only about 60 to 70% after 14 days (Novitsky 1986). Thus DNA-based qPCR assays might overestimate the concentration of viable cells due to the persistence of DNA from dead cells (Bae & Wuertz 2009). Even with this shortcoming, qPCR is an extensively used laboratory technique because it is not culture based.

Several molecular studies have found evidence of fecal human pathogens in rainwater (Ahmed et al. 2014, Ahmed et al. 2011, Ahmed et al. 2008). For example, Simmons et al. (2001) found *Cryptosporidium* spp. in 4 of 50 rainwater cistern samples in New Zealand. Ahmed et al. (2008) found genera containing fecally derived potential pathogens in cistern rainwater, such as *Campylobacter* spp. (12 of 27 samples), *Salmonella* spp. (3 of 27 samples), and *Giardia* spp. (4 of 21 samples) in Australia. While much attention has been paid to fecally derived potential human pathogens in harvested rainwater, non-fecal potential human pathogens also are of interest, including *Legionella pneumophila,* nontuberculous mycobacteria (NTM), and *Aspergillus* spp.

L. pneumophila can cause legionellosis, which commonly takes the form of the more serious Legionnaires' disease or the milder Pontiac Fever (Hicks et al. 2011, Kool et al. 1999). The presence of *L. pneumophila* in rainwater cisterns is problematic because Legionnaires' disease is transmitted by aerosol inhalation, and rainwater is widely used for cooling, irrigation, toilet flushing, car washing, and showering, all of which will generate aerosols. *L. pneumophila* lives in symbiosis with other microorganisms; *L. pneumophila* can colonize free-living protozoa (e.g., amoebae) such as *Acanthamoeba* spp., where the protozoa protect intracellular bacteria from adverse conditions or biocide treatments (Berjeaud et al. 2016, Thomas & Ashbolt 2010). Legionellosis outbreaks are correlated with the presence of biofilm (Abdel-Nour et al. 2013, Stout et al. 1985). Thus, rainwater

cisterns with biofilms in the sediment or in other locations of the RWH system could be a natural reservoir for *L. pneumophila* (Kobayashi et al. 2014, Kool et al. 1999).

The *mip* gene from *L. pneumophila* has been detected in harvested rainwater in several studies (Kobayashi et al. 2014, Ahmed et al. 2014). An outbreak of Legionnaires' disease was reported in New Zealand, where molecular analysis demonstrated that the *L. pneumophila* serogroup (Sg) 1 isolate from the respiratory tract of one of the people with Legionnaires' disease was indistinguishable from the *Legionella* isolates found in that person's RWH system as well as those from four other local RWH systems (Simmons et al. 2008).

NTM primarily cause pulmonary disease in immunocompetent people and bacteremia and disseminated infection in immunocompromised people (Falkinham 1996). Several studies suggest that treated drinking water is a source of human exposure to NTM (Covert et al. 1999, von Reyn et al. 1994). In an Australian study, serotypes of *Mycobacterium avium* and *Mycobacterium intracellulare* associated with disease were isolated in 20% of the 205 rainwater cisterns sampled (Tuffley & Holbeche 1980).

Aspergillus spp. are another type of waterborne potential human pathogen, but their occurrence in harvested rainwater has not yet been investigated. Some species of the fungus *Aspergillus* can cause aspergillosis, which ranges in severity from allergic respiratory symptoms to tissue or organ damage, often of the lungs. Aspergillosis is commonly caused by *Aspergillus fumigatus* and *Aspergillus flavus*; otomycosis, an infection of the outer ear canal, is commonly caused by *Aspergillus niger* (Bodey & Vartivarian 1989). *A. fumigatus* has been found in municipal tap water (Vesper et al. 2007), but no data exist on the occurrence of this or other fungi in harvested rainwater.

Overall, potential human pathogens appear to be relatively common in harvested rainwater. Consumers might incur health risks by indoor domestic use of harvested rainwater, if those waters are not suitably treated. Thus, it is important to understand the efficacy of harvested rainwater treatment.

2.4. TREATMENT OF HARVESTED RAINWATER FOR POTABLE USE

Several studies urge harvesters to disinfect their rainwater prior to potable use to improve microbiological water quality (Ahmed et al. 2014, Ahmed et al. 2012, Ahmed et al. 2011, Ahmed et al. 2010b, Schets et al. 2010).

Harvested rainwater treatment for potable use in the United States

Many RWH systems in the United States have filtration and disinfection systems. With respect to filtration, the ARCSA-member survey showed that most individual respondents with potable RWH systems use cartridge filters or activated carbon filters (Thomas et al. 2014). With respect to disinfection methods in potable RWH systems, the same survey found that UV light is the most common disinfection strategy (70%), with chlorination being used in a smaller number of systems (5%) for the surveyed population (Thomas et al. 2014). Filtered water passes through the UV chamber in a few seconds (Figure 2.3). UV manufacturers provide their own disinfection efficiency ratings, but field data from real RWH systems are sparse. Chlorination of harvested rainwater is usually performed directly in the cistern (batch chlorination). It is affordable, but there are risks of bacterial regrowth and disinfection byproduct formation when chlorine reacts with organic matter in the cistern (Keithley 2012, Helmreich & Horn 2009).

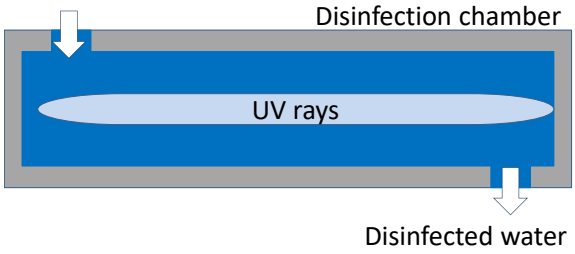

Figure 2.3: Schematic of ultraviolet (UV) system.

Other treatment techniques

Pasteurization by solar UV and slow sand filtration are cheap methods to improve the quality of harvested rainwater (Helmreich & Horn 2009). Solar disinfection (SODIS), which uses solar energy to inactivate microorganisms in water, has been studied for point-of-use rainwater treatment in South Korea (Amin & Han 2009). Metal membrane filters (1 -5 μm) and rotating disc filters with a ceramic membrane (0.06 μm) are expensive but suitable for rainwater treatment (Helmreich & Horn 2009).

2.5. THE MICROBIOME OF HARVESTED RAINWATER BEFORE AND AFTER TREATMENT

As reviewed in previous sections, harvested rainwater contains high numbers of unidentified bacteria, and the occurrence and concentration of indicator bacteria are not well correlated with the occurrence and concentration of potential human pathogens (Ahmed et al. 2014). Even though high concentrations of heterotrophic bacteria have been found in harvested rainwater, the microbial community of harvested rainwater has not been thoroughly described. To address this knowledge gap, the microbiome – which is the collection of genomes in an environment (Turnbaugh et al. 2009) – of rainwater should be

delineated. Interrogation of the microbiome of harvested/treated rainwater could provide insights into the design and management of rainwater harvesting systems.

Culture-based community analysis

Evans et al. (2009) cultured and isolated bacteria from 22 RWH cisterns; they found that there were more than 200 species and 80 genera in the cisterns, indicating the diverse microbial ecology of the rainwater cistern. However, culture-based microbial community analysis generally provides a narrow description of the microbial community because it is estimated that most microorganisms (up to 99.8%) are not culturable in the laboratory (Streit & Schmitz 2004). By contrast, DNA-based analysis can provide more detailed information about a microbiome.

DNA-based community analysis

Denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) were early DNA-based methods that were used to describe microbial community dynamics in various systems. DGGE followed by band sequencing was used to analyze microbial communities in several locations in a rainwater cistern (Kim & Han 2011); clear differences were observed between the planktonic and biofilm bacterial communities. This study also showed that most (88%) of the identified species were non-pathogenic Proteobacteria. However, such fingerprinting methods only show a limited number of taxa in a sample (Kim et al. 2013).

More recently developed 454 pyrosequencing and Illumina® sequencing are high-throughput, next-generation methods that have enabled thousands or millions of sequences to be acquired within a short analytical time and at a relatively low cost (Chidamba &

Korsten 2015, Kim et al. 2015, Navas-Molina et al. 2013, Caporaso et al. 2010). Illumina® Miseq followed by Quantitative Insight into Microbial Ecology (QIIME) can provide detailed taxonomic and phylogenetic information about a microbiome (Caporaso et al. 2010) to the genus level, which allows fine-scale microbiome descriptions.

Many efforts have been directed at understanding various microbiomes such as those of the human body, indoor environment, soil, and oceans (Moissl-Eichinger et al. 2015, Navas-Molina et al. 2013, East 2013, Kuczynski et al. 2010). In the field of drinking water, the microbiomes of municipal water distribution systems were studied using next-generation sequencing methods (Ji et al. 2015, Pinto et al. 2012), but very few non-culture/DNA-based microbiome analyses of harvested rainwater have been published (Chidamba & Korsten 2015, Cho & Jang 2014). Chidamba & Korsten (2015) used pyrosequencing targeting the V1–V3 hypervariable region of the 16S rRNA gene to describe the microbiome of untreated harvested rainwater in South Africa. They found that Proteobacteria dominated the rainwater microbiome. They also found the signatures of potential pathogens including *Legionella* at the genus level, where the relative abundance of *Legionella* spp. in rainwater was higher than that in river samples. Cho & Jang (2014) stored captured rainwater for 13-18 d at room temperature to examine how the rainwater community changes over time, using pyrosequencing of the V1–V3 region of the 16S rRNA gene. The authors found that the abundance of rare taxa decreases after storage. However, to the best of my knowledge, next-generation sequencing methods have not been used to compare the microbiome of harvested rainwater before and after residential-scale treatment.

2.6. FACTORS THAT CAN IMPACT THE RAINWATER MICROBIOME

Physicochemical conditions can impact the microbiomes of natural and engineered systems. For harvested rainwater, the dynamics of the cistern microbiome also will be impacted by physicochemical conditions, where some of these conditions might be under the control of the rainwater harvester.

Temperature is an important physicochemical factor that shapes the microbiomes of natural systems. For example, spatial censuses of hot springs found that temperature (53 - 93 ^{o}C) is an important factor that shapes the microbiome (Wang et al. 2014). The community richness (α-diversity) of soil samples was lower when the temperature was below freezing as compared to when it was above freezing (Smit et al. 2001). Additionally, the numbers of psychrophylic and mesophylic isolates from the sediment-water interface changed as a function of seasonal temperature (Ferroni & Kaminski 1980). The community richness of marine samples showed cyclical patterns based on season factors, such as day length, average temperature, and radiation (Gilbert et al. 2012). DOC is another parameter that can affect microbiome composition. Hot springs with different DOC concentrations (e.g., of 1.5 - 127.5 mg/L) had different microbiomes (Wang et al. 2014). Lian et al. (2017) also found that the soil microbiome composition appeared to be strongly linked to soil organic carbon.

It has been suggested that pH is the strongest physicochemical predictor of overall community composition. Fierer & Jackson (2006) showed that the soil bacterial community was richest (α-diversity) at neutral soil pH. The same study also showed that communities have similar composition if their pH is similar (β-diversity), even if the soil samples were taken at two different locations.

Many studies in the literature have addressed the factors that shape the microbiomes of engineered systems. Pinto et al. (2012) suggested that bacterial communities in drinking-water biofilters can be manipulated through various operational strategies, such as pH adjustment; the authors suggested that, in this fashion, beneficial microorganisms can be selected over harmful microorganisms. It also was found that the activated sludge reactor microbiome is constrained by biochemical oxygen demand (BOD), dissolved oxygen (DO), and temperature (Kim et al. 2013). Another study showed that community richness (α-diversity) was associated with ambient nitrogen and carbon availability, which is consistent with basic ecological principles where competition for growth-limiting resources affects diversity and richness (Johnson et al. 2014).

Other than physicochemical conditions, microorganisms already established in a microbial community can impact the future microbiome by selectively killing incoming microorganisms. In other words, bacteria defend their domain by recognizing and killing intruders. For example, when *E. coli* was introduced into an oral-derived bacterial community, it survived poorly (He et al. 2010); the same study found that oral-derived bacteria sense the lipopolysaccharides of *E. coli* and produce free radicals in response. It is unknown if a similar defense process would occur in a rainwater cistern microbiome that is disturbed by the entry of microorganisms in fresh rainfall.

The impact of physicochemical conditions in a rainwater cistern on the microbiome has not yet been explored. Since rainwater harvesters can exert control over some physicochemical conditions in the cistern (e.g., pH, presence of sediments, chlorine concentration, and possibly DOC concentration), the impact of these physicochemical factors must be studied.

3. IMPACT OF RESIDENTIAL TREATMENT PROCESSES ON HARVESTED RAINWATER QUALITY[2]

3.1. PROBLEM STATEMENT AND OBJECTIVES

Despite the existence of potential human pathogens in harvested rainwater (Ahmed et al. 2011), federal water quality regulations do not exist for potable rainwater harvesting (RWH) systems at individual residences in the United States. A survey of American Rainwater Catchment Systems Association (ARCSA) members showed that approximately 70% of individual respondents conduct water quality testing at least annually, while less than 13% of those surveyed test water quality on a quarterly basis (Thomas et al. 2014). While a plethora of studies have examined raw harvested rainwater quality, few data are available on the quality of harvested rainwater after treatment at individual RWH systems (Ahmed et al. 2012), particularly for the concentrations of potential human pathogens such as *Legionella pneumophila*, nontuberculous mycobacteria (NTM), and *Aspergillus* spp. Consequently, consumers of harvested rainwater might incur health risks, such as gastrointestinal illness, by consuming under-treated rainwater. Thus, examining rainwater quality in cisterns and treated tap water is important for understanding the efficacy of harvested rainwater treatment at individual residences and for assessing possible health concerns.

This chapter addresses **Task 1: Impact of residential treatment processes on harvested rainwater quality**. Here, harvested rainwater quality was surveyed from six full-scale residential RWH systems (five of which treat for potable use) in central Texas, where the systems are located within a 1-km radius of one another, as a case study. In this

[2] Largely taken from the following publication with permission of the publisher:
Kim, T, Lye, D., Donohue, M., Mistry, J. H., Pfaller, S., Vesper, S., and Kirisits, M. J. (2016) Harvested Rainwater Quality Before and After Treatment and Distribution in Residential Systems, *Journal AWWA*, 108 (11);

task, potable treatment consisted of chlorine disinfection followed by filtration or filtration followed by ultraviolet (UV) disinfection, and non-potable treatment consisted of filtration only. The specific research questions addressed were as follows:

1. How does residential-scale treatment change the physical, chemical, and microbiological quality of harvested rainwater?
2. Are indicator bacteria or heterotrophic plate counts (HPC) correlated with potential pathogens in harvested rainwater?
3. What physicochemical parameters are correlated with the microbiological quality of harvested rainwater?

3.2. MATERIALS AND METHODS

3.2.1. Site, sampling locations, sampling dates, and procedure overview

Six residential RWH systems in central Texas, located within a 1-km radius of one another, were selected for this study. Table 3.1 summarizes information about each system. The roof age was 2-22 years (avg. 12 years), roof areas were 1,300—5,400 ft^2 (avg. 3,000 ft^2), and total cistern volumes were 8,500 – 30,000 gallons (avg. 15,800 gal). Five systems (sites 1-5) had Galvalume® (metal) roofs and sites 1-4 had fiberglass cisterns (Figure 3.1).

Table 3.1: Key features of the rainwater harvesting systems

	Site number	1-Cl_2	2-UV	3-UV	4-UV	5-UV	6-NoDisinf
Roof	Material	Galvalume®	Galvalume®	Galvalume®	Galvalume®	Galvalume®	Asphalt fiberglass shingles
	Age (years)	22	17	15	14	1.5	2
	Footprint area (ft^2)	2,500	1,300	3,200	3,100	5,400	2,200
	Direction	Northeast /Southwest (NE/SW)	Northwest /Southeast	NE/SW	NE/SW	NE/SW	NE/SW
Gutter	Material	Aluminum	Aluminum	Polyvinyl chloride	Galvalume®	Aluminum	Polyvinyl chloride
Cistern	Inner-material	Fiberglass	Fiberglass	Fiberglass	Fiberglass	Polyethylene	Polyvinyl chloride
	Location	Above ground					
	Effluent pipe to house	Few inches above cistern bottom					
Treat-ment	First flush	Yes	Yes	No	Yes	Yes	No
	Roof-wash filter	No	Yes	Yes	Yes	Yes	No
	Recirculation filter	No	No	No	No	Yes	No
	Filter pore sizes (μm)*	30/5	info not available/5	5/3	5/3	50/5	2/2
	Disinfection	Chlorination	UV[a]	UV[a]	UV[a]	UV[b]	None
	Potable use	Yes	Yes	Yes	Yes	Yes	No
Piping	Materials	PVC (outside home) and copper (inside home)					
Cistern sampling point for this study		Few inches above bottom	Few inches above bottom	Few inches above bottom	Water surface	Water surface	Few inches above bottom

*All the sites have two filters between the cistern and tap

[a]UV output: 13.8 W (Maximum rated output at 254 nanometers), delivers a dose exceeding 40 mJ/cm^2 at a flowrate of 12 gal/min

[b]UV output: 19.3 W (Maximum rated output at 254 nanometers), delivers a dose exceeding 40 mJ/cm^2 at a flowrate of 12 gal/min

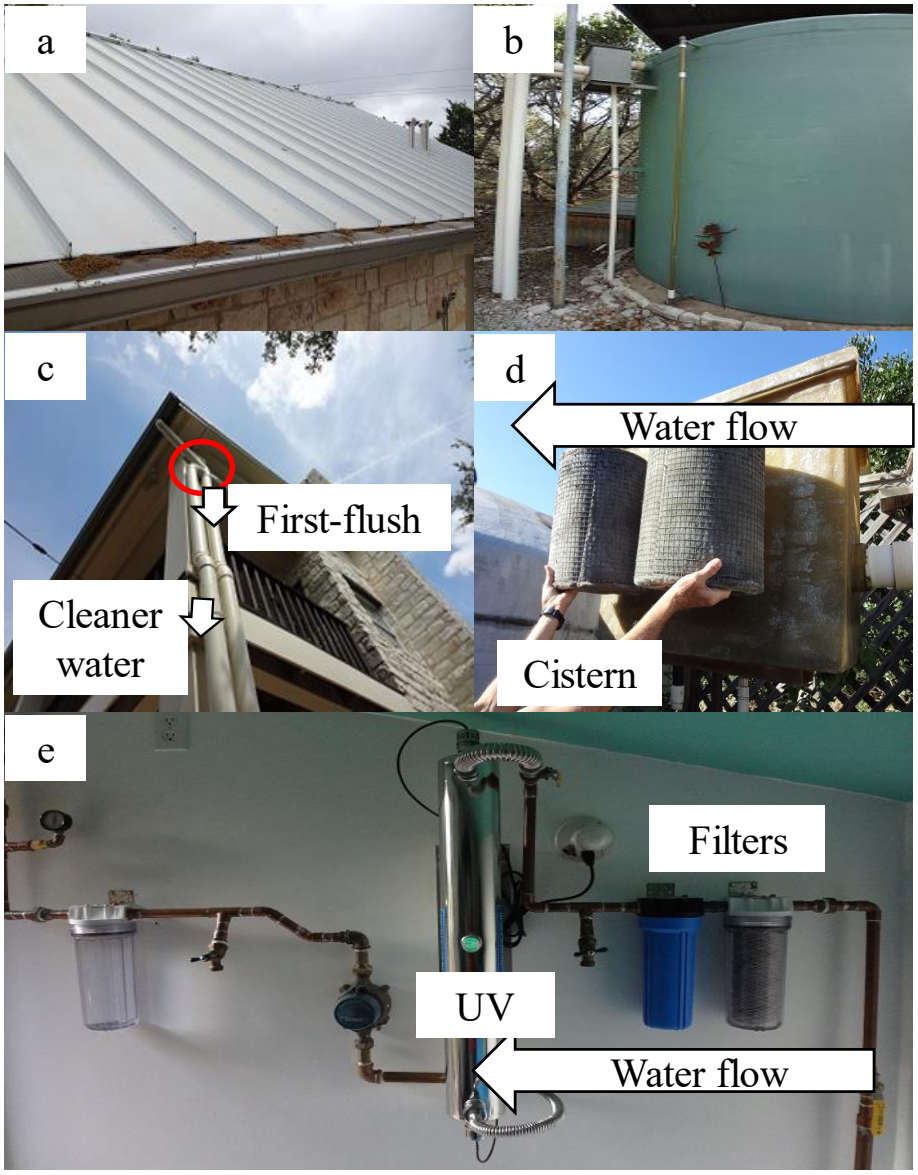

Figure 3.1: Pictures from several of the rainwater harvesting systems assessed in this study: (a) Galvalume® (metal) roof, (b) fiberglass cistern, (c) first-flush diverter (rainfall goes into the right pipe first; once it fills the pipe, then rainwater flows into the left pipe, (d) roof-wash filters, and (e) filtration/ultraviolet (UV) treatment system.

For disinfection, site 1-Cl_2 performs batch-chlorination in the cistern, sites 2-5 use UV light, and site 6-NoDisinf has no disinfection. Sites 1-5 use their treated rainwater for potable and non-potable purposes, and site 6-NoDisinf uses the water only for non-potable purposes. Schematic diagrams of the systems at sites 1-Cl_2, 3-UV, and 6-NoDisinf are depicted in Figure 3.2. All the UV sites (sites 2-5) either have roof-wash filters (which are placed between the gutters and the first cistern such that the rainwater is filtered before entering the cistern; Figure 3.1d) or recirculating filters (which are sand filters through which the cistern water is occasionally passed). In particular, site 5-UV operates its recirculating filter once per day. Four sites have first-flush diverters, which divert a fraction of the initial rainfall to a separate system (e.g., pipe or bath; Figure 3.1c), because the first flush tends to have higher contaminant concentrations as compared to subsequent volumes of harvested rainwater (Mendez et al. 2011). Each of the tested systems has two filters between the cistern and tap, with varying pore sizes (Table 3.1). Sampling events were conducted in October 2012 (fall), January 2013 (winter), April 2013 (spring), and July 2013 (summer). At each site, ca. 11 L of rainwater were collected aseptically from the cistern and a cold finished-water tap in the house. Samples were transported to The University of Texas at Austin for analysis of physical and chemical parameters, HPC, and indicator bacteria. Samples for analyzing total trihalomethanes (TTHM) were shipped to the United States Environmental Protection Agency (USEPA) lab in Houston, TX. Other samples were shipped to the USEPA in Cincinnati, OH for analyses of *L. pneumophila*, NTM, and *Aspergillus*.

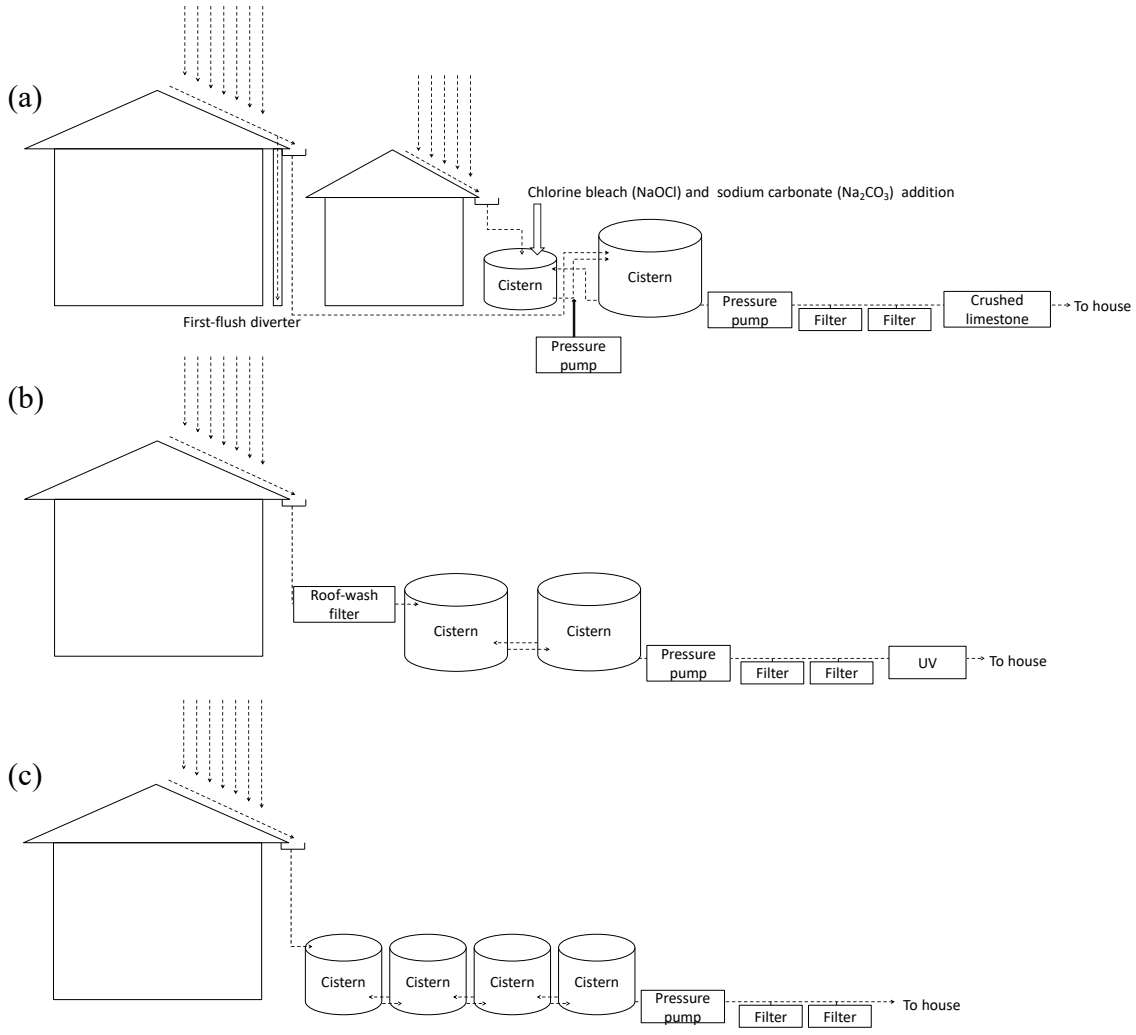

Figure 3.2: Rainwater harvesting system schematics for (a) site 1-Cl_2, (b) site 3-UV, and (3) site 6-NoDisinf; other UV sites (2-UV, 4-UV, and 5-UV) have the same disinfection procedure as at site 3-UV.

3.2.2. Physical and chemical analyses

Headspace-free samples were collected in 100-mL borosilicate glass bottles, which were used for residual chlorine (site 1-Cl_2 only), turbidity, pH, and dissolved oxygen (DO) measurements. Residual chlorine was measured using the Hach N,N-diethyl-p-

phenylenediamine (DPD) free chlorine reagent and a Hach Pocket Colorimeter™ II (Hach, Loveland, CO); turbidity was measured with a Hach 2100A Turbidimeter (Hach, Loveland, CO); pH was measured with an Orion 720A pH/ISE/mV meter (Thermo Fisher Scientific, Waltham, MA); DO was measured with a 54ABP DO meter (YSI Inc., Yellow Springs, OH). These analyses were performed within 6 hours of sampling. Accuracy, limit of detection (LOD), and precision for the analyses are reported in the Appendix. Dissolved organic carbon (DOC) samples were filtered through 0.45-μm nylon syringe filters (Thermo Fisher Scientific, Waltham, MA), acidified (to pH < 2) with phosphoric acid, and stored in 40-mL borosilicate vials with Teflon-lined septa. These were preserved at 4 °C for up to one month and measured using an Aurora 1030C TOC Analyzer (O.I. Corporation, College Station, TX). TTHMs from site 1-Cl_2 were measured once (April 2013). TTHM samples were dechlorinated with 25 mg of ascorbic acid in 40-mL borosilicate vials, acidified with hydrochloric acid (to pH < 2), and shipped overnight to the USEPA lab in Houston, TX, on ice. Upon receipt, the samples were analyzed by USEPA Method 524.2 (USEPA 1995).

3.2.3. HPC and indicator bacteria analyses

HPC were conducted according to Standard Methods for the Examination of Water and Wastewater 9215C (American Public Health Association (APHA) 2004). Samples were serially diluted in phosphate-buffered saline, plated on R2A agar (Becton Dickinson, Franklin Lakes, NJ), and incubated at 28 °C for 7 days. Indicator bacteria were enumerated by the most probable number (MPN) technique, utilizing Colilert® for total coliform (TC) and *Escherichia coli,* and Enterolert® for enterococci (IDEXX, Westbrook, ME). Quality control results for the MPN tests are reported in the Appendix.

3.2.4. Quantitative, real-time polymerase chain reaction (qPCR) analyses

For DNA-based analyses, rainwater samples were collected in 10-L sterile polypropylene bottles. For *Legionella* analyses, a portion of each sample was vacuum-filtered through a 0.22-µm polyethersulfone water filter (Mo Bio Laboratories, Carlsbad, CA) until the filter was clogged (median of 1.0 L). DNA was extracted from the filter using a RapidWater® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. For analyses of NTM (*Mycobacterium avium* and *Mycobacterium intracellulare*) and *Aspergillus* spp., 1 to 2 L of each rainwater sample were vacuum-filtered through a 0.45-µm polycarbonate filter (Osmonics, Inc., Minnetonka, MN). Each filter was placed aseptically into a 2-mL, sterile, screw-cap tube containing 0.3 grams of 0.1-mm sterile glass beads. An aliquot of the extracted DNA and the polycarbonate filters were shipped overnight on ice to the USEPA lab in Cincinnati, OH. Filters and DNA were kept at -20 °C until analysis.

qPCR assays were used to measure *L. pneumophila*, NTM, and *Aspergillus* spp. Although qPCR assays might overestimate the concentration of viable cells due to the persistence of DNA from dead cells (Bae & Wuertz 2009), similar DNA-based assays have been used in other rainwater quality surveys (e.g., Ahmed et al. 2014, Kaushik et al. 2014, Ahmed et al. 2008). Thus, they were employed here to complement the culture-based assays used for HPC and indicator bacteria.

Two *L. pneumophila* assays were employed. The Lp16S assay detects *L. pneumophila* at the species level, and the LpLPS assay detects *L. pneumophila* Sg 1; details of the procedure have been published previously (Donohue et al. 2014). Additionally, the *M. avium* and *M. intracellulare* qPCR assays were carried out according to published

protocols (Chern et al. 2015, Beumer et al. 2010), as were the *Aspergillus flavus*, *Aspergillus fumigatus*, and *Aspergillus niger* qPCR assays (Haugland et al. 2004).

3.2.5. Data analysis

Due to the small number of sampling events, median values of water quality parameters were calculated rather than mean values. To calculate conservative median or log-removal quantities, those samples that were below the LOD or limit of quantification (LOQ) were assigned a value exactly equal to the LOD or LOQ. Log removals were calculated for paired samples (i.e., the cistern and tap for a particular site and sampling event), and then the median log removal over all events was calculated. The nonparametric Mann-Whitney test, with a significance level of $\alpha = 0.05$ and two-tailed hypothesis, was adopted for statistical analyses to compare water quality data among sites and treatments (where at least 5 data points in each group were available).

To examine correlations between potential human pathogens and other water quality parameters at the site 2-5 cisterns (i.e., non-chlorinated cisterns fed by Galvalume® roofs; Table 3.1), Spearman's rank correlations were calculated. Of ten microbial parameters, only HPC, *A. fumigatus*, and *A. niger* were found in every sample at the site 2-5 cisterns. All non-detect samples were assigned a numerical value of 0 for rank calculations. When non-detects (of potential pathogens) were included in the rank calculations, two additional tests were performed: (1) another Spearman's rank calculation excluding the non-detect samples, and (2) a Mann-Whitney test, with a significance level of $\alpha = 0.05$ and one-tailed hypothesis, to determine if the dependent variable (HPC, TC, DOC, or pH) was significantly different in the presence of a specific potential pathogen

(e.g., *L. pneumophila*) in the cistern as compared to the absence of that pathogen. Statistical analyses were conducted in Minitab® (Minitab, State College, PA).

3.3. RESULTS

3.3.1. Physical and chemical water quality

The pH of harvested rainwater, in cisterns and at taps, often falls within the USEPA secondary drinking water standard of 6.5-8.5 (Figure 3.3a). The median pH (i.e., -log of median [H^+]) from sites 2-6 was 6.8 in both cistern and tap samples. The median pH at sites 1-Cl_2 and 3-UV are higher than at the other sites because of the addition of sodium carbonate at the cistern (Figure 3.3a) to prevent corrosion of metal pipes. As shown in Figure 3.3b, sites with roof-wash or recirculating filters (sites 2-UV, 3-UV, 4-UV, and 5-UV) had significantly lower turbidity in the cisterns (Median [*Mdn*] = 0.34 nephelometric turbidity units [NTU]) as compared to sites without them (*Mdn* = 2.88 NTU at sites 1-Cl_2 and 6-NoDisinf), $Z = 3.64$, $p = 0.0003$. After cartridge filtration at sites 2-5, the turbidity significantly dropped from the cistern (*Mdn* = 0.34) to the taps (*Mdn* = 0.24 NTU), $Z = 2.43$, $p = 0.02$; thirteen out of sixteen tap samples (81%) at those sites had turbidity ≤ 0.3 NTU, which is the USEPA primary drinking water standard for regulated treatment systems. Without roof-wash or recirculating filters (sites 1-Cl_2 and 6-NoDisinf), the median turbidity was 0.38 NTU at the taps; only three out of seven tap samples (43%) at those sites met the USEPA standard of 0.3 NTU.

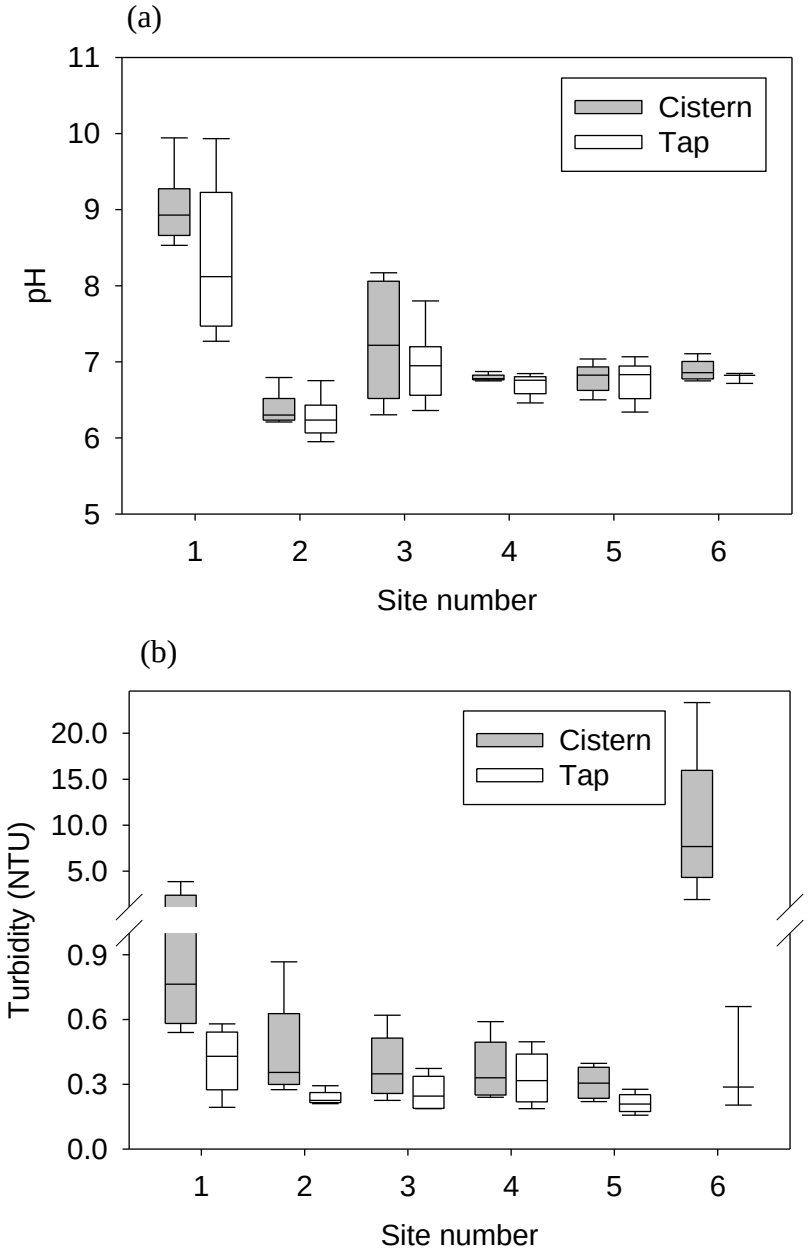

Figure 3.3: Physical and chemical water quality at each of the six sampled sites throughout the year, n = 4 sampling events, (a) pH and (b) turbidity.

* Lines in boxes represent median values, vertical boxes represent 25th and 75th percentiles, and error bars represent maximum and minimum.

The median DOC concentration from sites 1-5 (Galvalume® roofs) was 1.0 mg/L in the cisterns and 0.6 mg/L at the taps (Figure 3.4a), but the shingle roof at site 6-NoDisinf showed higher median DOC values (13.7 mg/L in the cistern and 6.2 mg/L at the tap). This is consistent with a previous study of the impact of roofing material on harvested rainwater quality, which showed that rainwater harvested following first-flush diversion from an asphalt fiberglass shingle roof yielded a higher DOC concentration as compared to that harvested from a metal roof (Mendez et al. 2011). Elevated DOC might contribute to TTHM generation after chlorination (Keithley 2012), so chlorine disinfection in conjunction with an asphalt fiberglass shingle roofing catchment should be carefully applied. At site 1-Cl_2, chlorine disinfection was performed after each substantial rainfall by dosing household bleach into the small cistern and recirculating this chlorinated water between the two cisterns (Figure 3.2a), so that ~1 mg/L free chlorine residual was detectable in the large cistern on the following morning. The free chlorine residual measured at site 1-Cl_2 during this study ranged from 0.05 (the LOD) to 2.2 mg/L at the tap. During the April 2013 sampling (when DOC was 0.9 mg/L at the site 1-Cl_2 cistern), the TTHM concentrations at site 1 were 19.2 μg/L at the cistern and 22.8 μg/L at the tap; these levels are below the 80 μg/L USEPA maximum contaminant level, suggesting that TTHM formation at this chlorinated Galvalume® site is not a cause for concern. Figure 3.4b indicates that all cisterns contained DO throughout the year. Thus, anaerobic bacteria are not expected in the bulk water and thereby would not impact water quality.

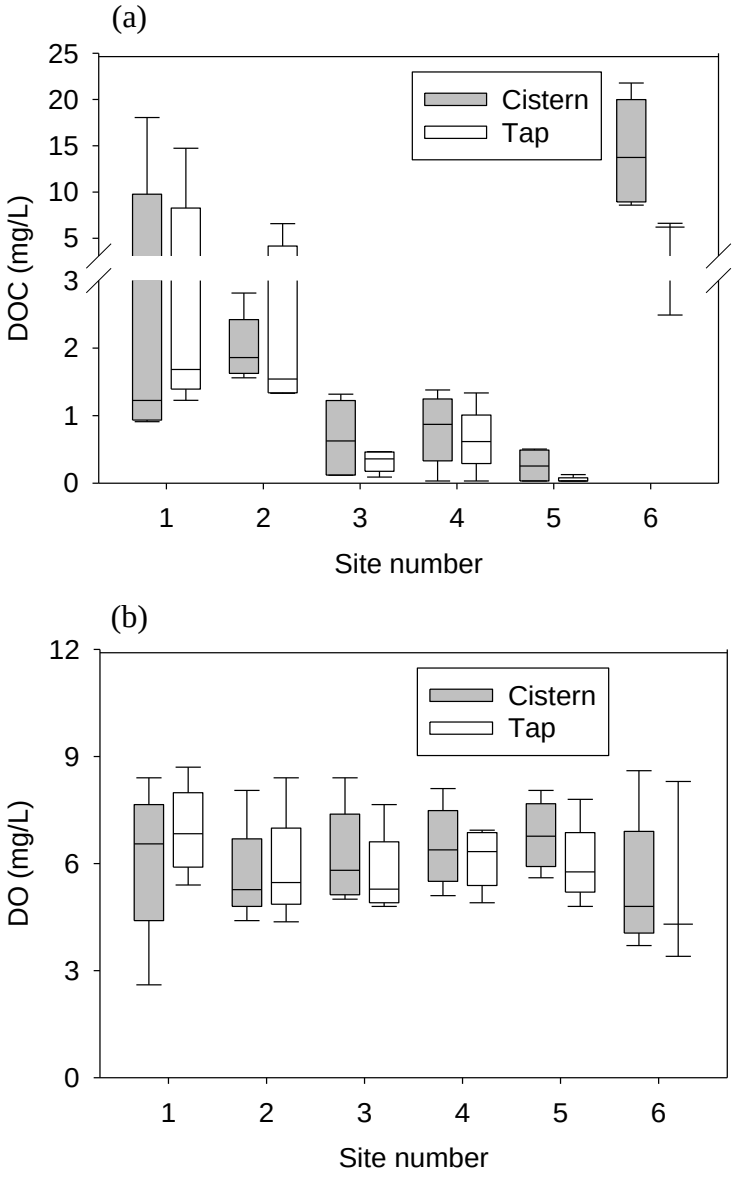

Figure 3.4: Physical and chemical water quality at each of the six sampled sites throughout the year, n = 4 sampling events, (a) dissolved organic carbon (DOC), and (b) dissolved oxygen (DO).

* Lines in boxes represent median values, vertical boxes represent 25th and 75th percentiles, and error bars represent maximum and minimum.

3.3.2. HPC and indicator bacteria

The HPC of chlorinated rainwater and UV-disinfected rainwater in this study (Figure 3.5) generally were in the range observed in community water systems. For instance, Carter et al. (2000) found log-mean HPC (colony-forming unit [CFU]/mL) = 2.6 in the distribution system in Milford, OH; in the current study, tap water in Austin, TX contained log-HPC (CFU/mL) = 3.9; Pepper et al. (2004) reported log-mean HPC (CFU/mL) = 5.5 at residential taps in Tucson, AZ. The chlorinated site (site 1-Cl_2) generally had lower HPC at the tap as compared to the taps at the UV-disinfected sites (sites 2-5) and the no-disinfection site (site 6-NoDisinf, Figure 3.5a). The four UV sites (sites 2-5) showed a median log-removal for HPC of 1.0 after filtration, UV treatment, and distribution to the sampled tap (Table 3.2); the HPC at these taps were significantly lower than those in the cisterns, $Z = 2.77$, $p = 0.03$. The no-disinfection site (site 6-NoDisinf) showed a median log-removal of 1.8 (Table 3.2) with only the use of two home-installed filters; it is possible that some of the microorganisms were attached to particles in the water that were removed during filtration (see substantial turbidity removal at site 6-NoDisinf, Figure 3.3b).

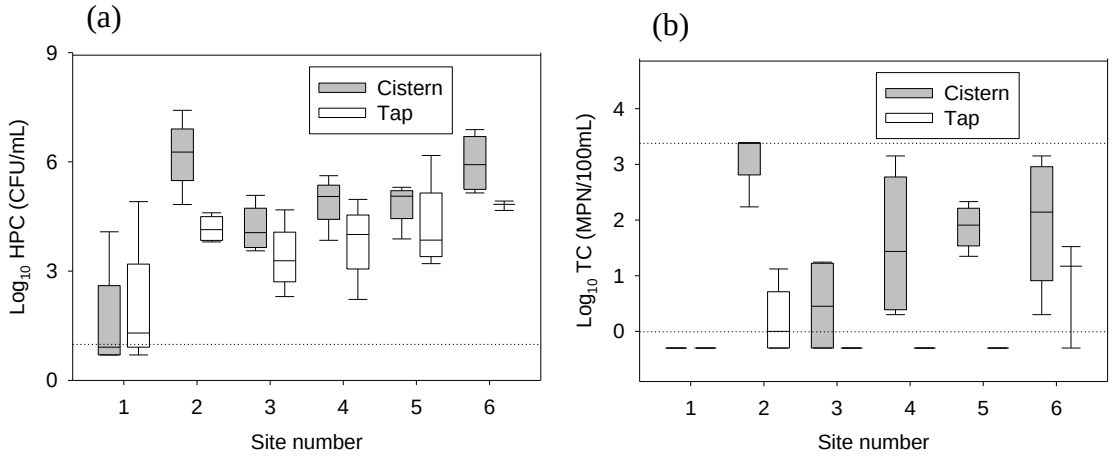

Figure 3.5: Microbiological water quality at each of the six sampled sites throughout the year, n = 4 sampling events, (a) heterotrophic plate counts (HPC) and (b) total coliform (TC).

Lines in boxes represent median values, vertical boxes represent 25th and 75th percentiles, error bars represent maximum and minimum, and dashed lines indicate the detection limit through the upper counting range. Non-detects were plotted as 5 CFU/mL or 0.5 MPN/100mL, which are one-half of the detection limit.

Table 3.2: Median log-removal of cultured organisms at each site through filtration/ultraviolet (UV)/distribution (site 2-UV through 5-UV) or through filtration/distribution only (site 6-NoDisinf)

Site number	Heterotrophic plate count (HPC)	Total coliform (TC)	*Escherichia coli*	Enterococci
2-UV	2.4	> 2.7[a]	2.3	> 2.5
3-UV	0.7	> 1.2	n/a[b]	> 0.6
4-UV	1.3	> 1.4	n/a	> 1.6
5-UV	0.8	> 1.9	>0.6	> 1.7
UV sites overall	1.0	> 2.2	> 1.4	> 1.7
6-NoDisinf	1.8	0.8	0.4	> 1.7

[a]greater-than sign (>) indicates that the log-removal calculation includes one or more tap samples that were below the limit of detection or limit of quantification.

[b]n/a: not detected at the cistern.

Indicator bacteria commonly occurred in the non-chlorinated cisterns (Figure 3.5b, Figure 3.6). For example, 18 out of 20 samples (90%) from non-chlorinated cisterns (sites 2-6) were positive for TC, and 14 out of 20 samples (70%) from non-chlorinated cisterns were positive for enterococci. Similar to the study of Ahmed et al. (2014) the frequency of occurrence of enterococci in the cisterns exceeded that of *E. coli*. Even though TC and enterococci were frequently found in non-chlorinated rainwater, their concentrations were not well-rank-correlated (Spearman's rho [r_s] = 0.18, p = 0.45). The results also showed that microbiological quality in the cistern, as measured by the concentrations of fecal indicator bacteria, can vary greatly from site to site (even though these six sites are located very close together). This suggests that the microbiological quality of harvested rainwater in one cistern cannot be anticipated from that of neighboring cisterns. The concentrations of indicator bacteria in harvested rainwater were low after disinfection with chlorine or UV light. TC and enterococci always were absent from the chlorinated site (site 1-Cl_2) at the cistern and tap. Of the 16 tap samples from UV sites (sites 2-5), two samples were positive with TC, but enterococci were not detected in any of those tap samples. *E. coli* were rare in cistern and tap samples; *E. coli* was absent from site 1-Cl_2, and only five out of 20 (25%) non-chlorinated cistern samples (sites 2-6) contained *E. coli*. In one case, *E. coli* was still found at the site 2-UV tap after filtration and UV treatment; the tap at this site also had back-to-back positive TC results. Together, those results suggest a deficiency in the filtration/UV process and/or degradation in water quality in the distribution system due to the absence of a disinfectant residual. Even though site 6-NoDisinf (no disinfection, but two filters) showed significant reduction in HPC due to filtration (Table 3.2), TC, *E. coli*, and enterococci were still detected at its tap (Figure 3.5, Figure 3.6). This finding

reconfirms that an adequate disinfection system, in addition to filtration, is needed to remove indicator bacteria from harvested rainwater.

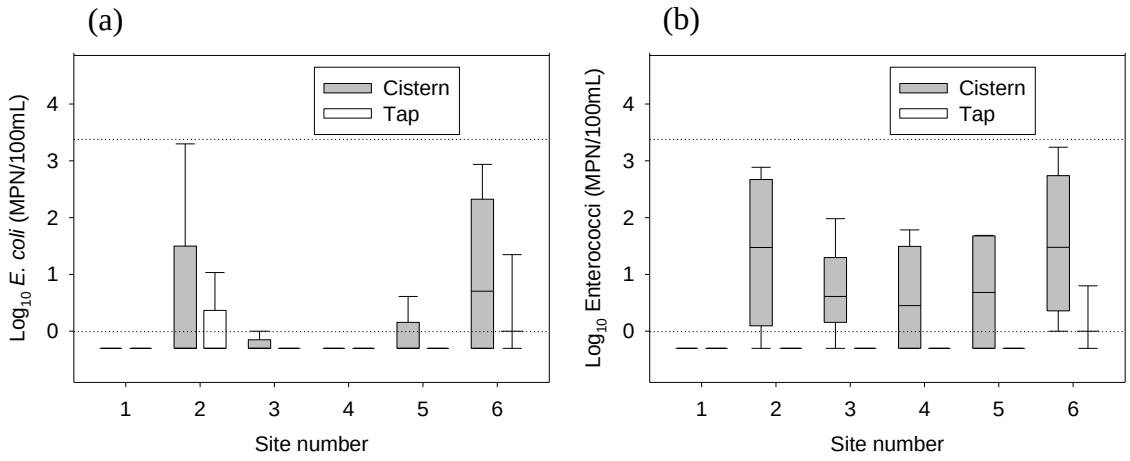

Figure 3.6: Microbiological water quality at each of the six sampled sites throughout the year, n = 4 sampling events, (a) *Escherichia coli* and (b) enterococci concentrations.

Lines in boxes represent median values, vertical boxes represent 25th and 75th percentiles, error bars represent maximum and minimum, and dashed lines indicate the detection limit through the upper counting range. Non-detects were plotted as 0.5 MPN/100mL, which is one-half of the detection limit.

The limited reduction in HPC from the cisterns to the taps (after filtration, UV treatment, and distribution) at sites 2-5 (Table 3.2) suggests deficiencies in the filtration/UV process and/or degradation in water quality in the distribution system due to the absence of a disinfectant residual. While disinfection efficacy of the filtration/UV processes in this study was not determined (due to lack of sampling ports just upstream and downstream of the treatment processes), inadequacies in disinfection could occurred for the following reasons. (1) The actual germicidal fluence of these UV treatment systems in the field might be less than their >40 mJ/cm^2 rating from the manufacturer. Since no UV

lamp maintenance (other than replacing the lamp) was reported by the harvesters in this study, scaling of the sleeve for the UV lamp would have gone unchecked, thereby reducing UV transmittance. On-site measurement of UV fluence is recommended at drinking water treatment plants (Hijnen et al. 2006), and a longitudinal study of fluence in a UV system for harvested rainwater would be prudent. (2) The environmental bacteria that persist on rooftops might have increased UV tolerance as compared to environmental bacteria in a surface water, which already have increased UV tolerance as compared to the lab strains typically used for UV studies (Hijnen et al. 2006). (3) Due to the absence of a disinfectant residual in the distribution system for the UV-treated sites (2-5), bacterial regrowth and sloughing in the distribution system could lead to higher microbial concentrations at the tap.

3.3.3. Quantification of *L. pneumophila* in harvested rainwater

Legionella spp. have been shown to occur in water treated with a variety of disinfectants. Donohue et al. (2014) observed that colonized cold-water taps (those showing more than one detection of *L. pneumophila* by qPCR) were found more frequently in chlorinated as compared to chloraminated drinking water systems. Additionally, greater culturable *Legionella* spp. were found in a water distribution system using chlorinated water as compared to UV-disinfected water (Långmark et al. 2005). In the current study, *L. pneumophila* genomic targets were detected in two cisterns (sites 2-UV and 3-UV) and at two taps (sites 1-Cl_2 and 2-UV) with the Lp16S assay (Figure 3.7). However, those samples were negative for *L. pneumophila* Sg 1, which caused 84% of legionellosis cases in 2009-2010 (Donohue et al. 2014). These data suggest that the *L. pneumophila*

populations at sites 1-3 are mostly composed of *L. pneumophila* Sg 2-15, which have lower risk of causing legionellosis.

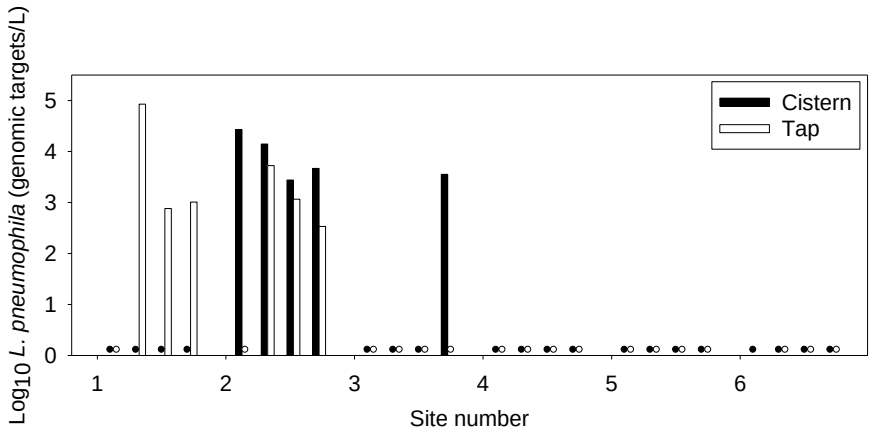

Figure 3.7: *Legionella pneumophila* concentrations at each of the six sampled sites throughout the year (with the Lp16S assay, which detects *L. pneumophila* at the species level).

Four sets of bars in series represent October 2012, January 2013, April 2013, and July 2013 samplings, respectively. Dots indicate non-detects; no tap sample was collected at site 6 in October 2012.

At the chlorinated site (site 1-Cl_2), *L. pneumophila* was observed at the tap but not at the cistern (Figure 3.7); this suggests that *L. pneumophila* has colonized the filters and/or distribution system between the cistern and tap at that site. The log-median *L. pneumophila* concentration in units of genomic targets/L was 2.9 at the site 1-Cl_2 tap. *L. pneumophila* also was detected in tap samples from municipal drinking water systems at $10^{1.7}$ CFU/mL by plate counting method (States et al. 1987), which is lower than the concentrations in the RWH system tap samples in this study. However, a discussed earlier, the use of a DNA-based method might overestimate the concentration of viable *L. pneumophila*. Therefore,

investigation of viable/culturable *L. pneumophila* concentrations in treated rainwater is needed to better understand consumer risk due to RWH for indoor domestic use.

The limited decreases in *L. pneumophila* concentrations from the cistern to the tap after filtration, UV treatment, and distribution at site 2-UV suggest deficiencies in the filtration/UV process and/or degradation of water quality in the distribution system due to the absence of a disinfectant residual. At site 2-UV, the log-median *L. pneumophila* concentrations were 4.0 at the cistern and 2.8 at the tap. These data contrast with the results of Hijnen et al. (2006), who used culture-based data from well-controlled lab studies to estimate that 30 mJ/cm^2 (low-pressure UV) is required for 4-log inactivation credit of environmental *L. pneumophila* strains. In addition to the three factors outlined in the previous section on HPC that might contribute to higher-than-expected microbial concentrations at the tap of the filter/UV-treated systems, the *L. pneumophila* concentrations measured in treated rainwater with qPCR might overestimate viable or culturable concentrations, which are of greater importance with respect to human disease.

Overall, the *L. pneumophila* genomic target concentrations found at the taps for sites 1 and 2 in the current study were higher than those at most of the cold-water taps at homes supplied with potable mains water (Donohue et al. 2014). Also, it appears that *L. pneumophila* tended to persist in a RWH system after its first detection.

3.3.4. Quantification of *M. avium* and *M. intracellulare* in harvested rainwater

M. avium was detected in 10 out of 24 (42%) cistern samples and 7 out of 23 (30%) tap samples (Figure 3.8a). Sites 3-UV and 5-UV cistern samples always were positive for *M. avium*, and the log-median concentrations (in units of genomic targets/L) were 3.9 and 3.3 at site 3-UV and site 5-UV, respectively. At site 6-NoDisinf, there were two cases

where *M. avium* was detected at the tap but not at the cistern. *M. intracellulare* was detected more frequently as compared to *M. avium*. *M. intracellulare* was detected in 19 out of 24 (79%) cistern samples and 16 out of 23 (70%) tap samples; furthermore, it was detected at every site at least once (Figure 3.8b). *M. intracellulare* was found consistently in the chlorinated cistern (site 1-Cl_2). Given their hydrophobicity, mycobacteria are quite resistant to disinfection and tend to persist in treated drinking water. For instance, NTM were found in drinking water samples with an average free chlorine concentration of 0.7 mg/L (Covert et al. 1999). In the current study, the site 1-Cl_2 tap (chlorination/filtration) showed a log-median *M. intracellulare* concentration of 1.0, while the site 2-5 taps (filtration/UV) showed a log-median *M. intracellulare* concentration of 2.3. Log-removal values from the cisterns to the taps after filtration, UV treatment, and distribution varied; median log-removals at sites 2-UV and 3-UV were both 0.6, and median log-removal at site 5-UV was > 2.2. Interestingly, *M. intracellulare* concentrations increased from the cistern to the tap at site 4-UV at every sampling (Figure 3.8b), but a specific characteristic of site 4-UV that would contribute to amplification of *M. intracellulare* in the system was not identified. Other studies have found increases in NTM from the drinking water treatment plant to the tap (e.g., Hilborn et al. 2006). Falkinham et al. (2001) recovered high concentrations of *M. intracellulare* in distribution system biofilms and showed that biofilms can act as a reservoir of mycobacteria.

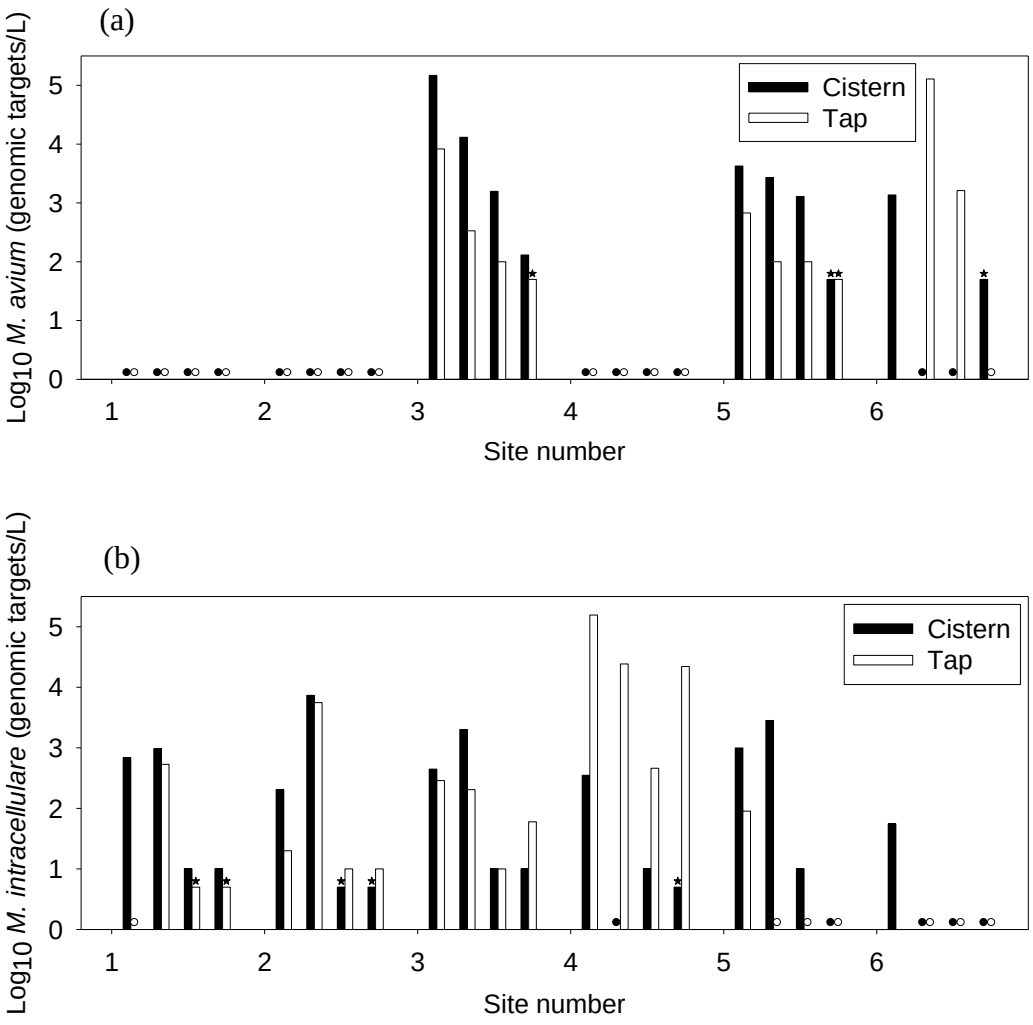

Figure 3.8: (a) *Mycobacterium avium*, and (b) *Mycobacterium intracellulare*. concentrations at each of the six sampled sites throughout the year.

Four sets of bars in series represent October 2012, January 2013, April 2013, and July 2013 samplings, respectively. Dots indicate non-detects; detections below the limit of quantification (LOQ) were assigned a numerical value of 50 or 5 genomic targets/L, which are one-half the LOQs, and marked with an asterisk. No tap sample was collected at site 6-NoDisinf in October 2012.

The data show that substantial quantities of environmental mycobacteria (as measured by genomic targets) remain in treated harvested rainwater at the tap. While Hayes et al. (2008) demonstrated 4-log inactivation of mono-disperse *M. avium* and *M.*

intracellulare with UV exposures < 20 mJ/cm^2 using a low-pressure UV lamp, the environmental mycobacteria in the current study were likely protected from disinfection due to clumping or cording. Additionally, the higher-than-expected NTM concentrations at the tap in this study could have been due to any of the four reasons outlined in the previous HPC and *L. pneumophila* sections. The data suggest that consumers at RWH sites might be at risk of NTM exposure from their tap water, but it is unknown if their exposure risk exceeds that of consumers supplied with mains water. The frequency of detection of *M. avium* and *M. intracellulare* at taps in RWH systems in this study is not unlike the frequencies detected at taps in homes supplied with potable mains water in the United States; for example, 54% tap samples were positive for *M. avium* in the study conducted by Hilborn et al. (2006) as compared to 30% of tap samples in the current study.

3.3.5. Quantification of *Aspergillus* species in harvested rainwater

The results demonstrate that opportunistic pathogenic *Aspergillus* spp. can be found in high concentrations in rainwater in the cistern and at the tap (Figure 3.9). Despite substantial declines in genomic targets of *Aspergillus* spp. from the cisterns to the taps after filtration, UV treatment, and distribution (sites 2-5), *A. fumigatus* and *A. niger* were detected commonly at the finished-water taps. Nourmoradi et al. (2012) observed four-log removal of *A. niger* by UV treatment at 16.6 mJ/cm^2, with culture-dependent quantification. The higher-than-expected *Aspergillus* species concentrations at the taps in this study could have been due to any of the four reasons outlined in the previous HPC and *L. pneumophila* sections. Filamentous fungi, such as species of *Aspergillus*, are common heterotrophs in nature, but the public risk from ingestion/exposure to these levels of *Aspergillus* in water for indoor domestic use remains unknown.

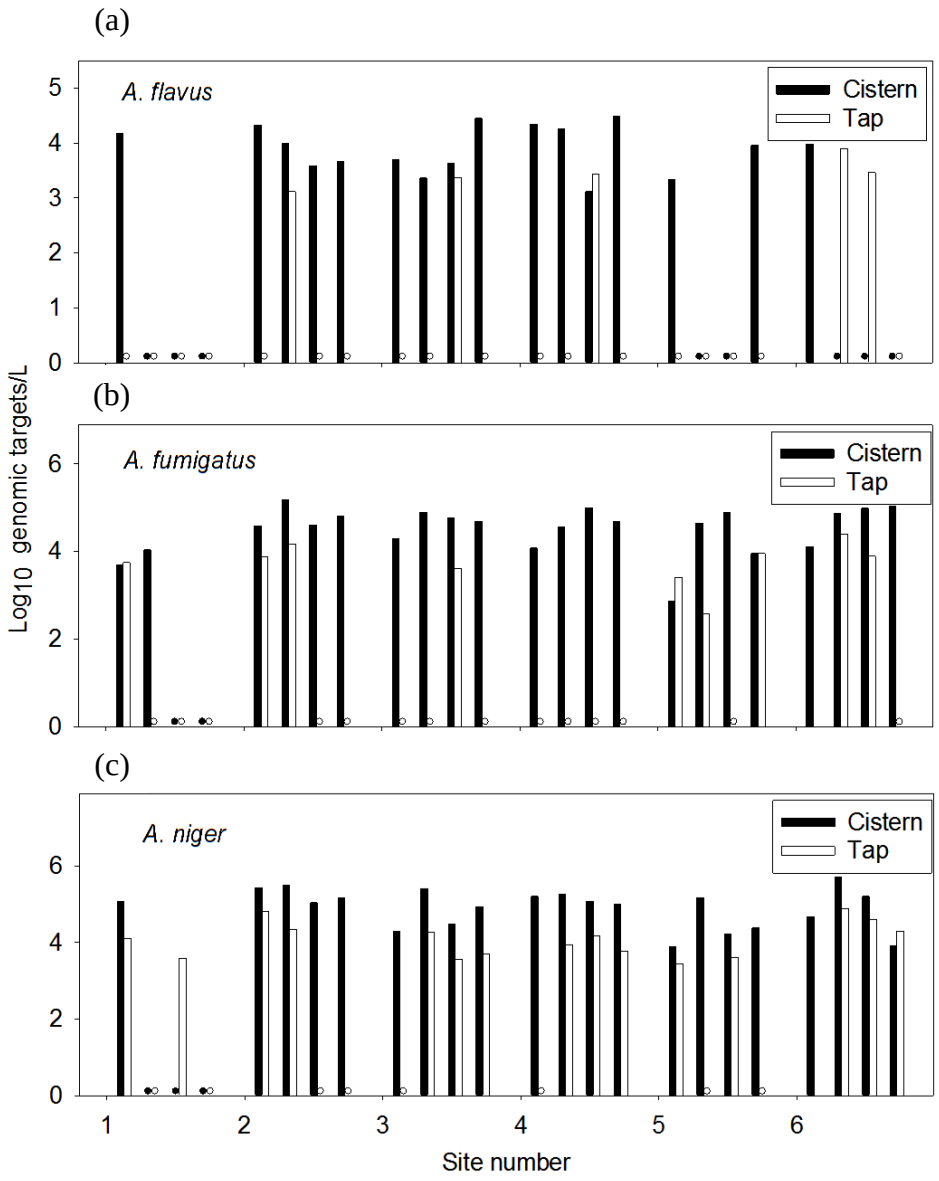

Figure 3.9: Potential human pathogenic fungi concentrations at each of the six sampled RWH sites throughout the year (a) *Aspergillus flavus*, (b) *Aspergillus fumigatus*, and (c) *Aspergillus niger*.

Four sets of bars in series represent October 2012, January 2013, April 2013, and July 2013 samplings, respectively. Dots indicate non-detects. No tap sample was collected at site 6-NoDisinf in October 2012.

3.3.6. Drawing correlations between water quality parameters at RWH systems with filtration/UV treatment

Given that most of the RWH systems tested in this study included filtration/UV-treatment, those data were compiled to examine potential correlations between water quality parameters. As discussed below, correlations were tested at the non-disinfected cisterns and at the finished-water taps for sites 2-UV through 5-UV.

As expected, no positive correlations were observed between the non-fecal potential pathogens examined in this study (i.e., *L. pneumophila*, *M. avium*, *M. intracellulare*, *A. flavus*, *A. fumigatus*, and *A. niger*) and typical fecal indicator bacteria (i.e., TC, *E. coli*, and enterococci). Further, these potential pathogens were sometimes detected in treated rainwater samples via DNA-based methods even when no indicator bacteria were detected via MPN analyses. Thus, consistent with the rainwater literature, these data suggest that the use of fecal indicator bacteria is insufficient to alert the harvester to the presence of potential human pathogens in rainwater.

DOC concentration was positively rank-correlated with HPC, *L. pneumophila* concentration, and *A. niger* concentration with a significance level $p \leq 0.05$ (Table 3.3). The potential for limiting the occurrence/concentration of potential pathogens such as *L. pneumophila* and *A. niger* in harvested rainwater by decreasing the DOC input to the cistern (e.g., via removal of overhanging vegetation and regular gutter cleaning) should be evaluated.

Table 3.3: Correlation between water quality parameters of 16 samples from cisterns at sites 2-5 (non-chlorinated cisterns where water is collected from Galvalume® roofs)

Concentration vs. Concentration	Spearman's rho (r_s)	p
Heterotrophic plate count (HPC) vs. dissolved organic carbon (DOC)	0.49	0.05
HPC vs. *Legionella pneumophila*	0.58	0.04
DOC vs. *L. pneumophila*	0.61	0.01
Aspergillus niger vs. DOC	0.67	0.01
HPC vs. *Mycobacterium avium*	-0.51	0.04
Total coliform (TC) vs. *M. avium*	-0.55	0.03
pH vs. *M. avium*	0.52	0.04

*Only notable correlations are shown, and the Spearman's rank calculation included each non-detect at the lowest rank.

pH was positively rank-correlated with the concentration of *M. avium* (Table 3.3), and *M. avium* was consistently found at site 3-UV where the homeowner added sodium carbonate to the cistern (which resulted in a higher pH than in the other non-disinfected cisterns). The *M. avium* concentration was negatively rank-correlated with HPC and TC concentration (Table 3.3). From 20 non-disinfected cistern samples, TC was absent only twice at site 3-UV, and those two cases were when the cistern pH was higher than 7.9. Thus, pH also might impact TC survival.

3.3.7. Conclusions and succeeding task

Conclusions

In this chapter, rainwater that was harvested, treated (chlorine disinfection followed by filtration, filtration followed by UV disinfection, or filtration only), and distributed at six residential sites for indoor domestic use was surveyed for its quality. Those measurements included temperature, pH, turbidity, DOC, DO, residual chlorine, TTHMs,

HPC, indicator bacteria (TC, *E. coli*, and enterococci), and potential human pathogens (*L. pneumophila*, *M. avium*, *M. intracellulare*, *A. flavus*, *A. fumigatus*, and *A. niger*). The following conclusions were drawn:

1. The low decreases in bacterial concentrations from the rainwater cistern to the tap (after filtration, UV treatment, and distribution) suggest that more conservative design, operation, and maintenance guidelines are needed for safe potable RWH. In particular, a study of longitudinal fluence in residential-scale UV systems and the associated maintenance of those systems is needed; additionally, the impact of having no disinfectant residual in the distribution system following UV treatment must be assessed for rainwater.
2. RWH systems that are located geographically close to one another will not necessarily have similar cistern water quality. Neither will those systems necessarily yield similar tap water quality, even if they have similar treatment processes in place. Overall, harvested rainwater quality cannot be anticipated from neighboring systems because system history, design, operation, and maintenance are likely to strongly impact water quality.
3. The RWH site practicing batch chlorination in the cistern generally had lower HPC at the finished-water tap as compared to that at the UV-disinfected sites (log median [CFU/mL] of 1.3 and 3.9, respectively). Additionally, fecal indicator bacteria always were absent after chlorination of the harvested rainwater, which is expected under proper disinfection conditions. However, potential human pathogens still were detected in the chlorinated system by DNA-based methods.
4. *L. pneumophila* Sg 1-15 were not observed in the chlorinated rainwater cistern but Sg 2-15 were detected downstream at the tap by DNA-based methods. This

suggests that the system downstream of the chlorinated cistern might be colonized by *L. pneumophila*, which could act as a long-term reservoir of *Legionella*.

5. Mycobacteria, which are hydrophobic and resistant to disinfection, were detected frequently by DNA-based methods at the finished-water taps for chlorinated and UV-disinfected systems. However, the frequency of occurrence of *M. avium* and *M. intracellulare* at taps in RWH systems in this study is not unlike the frequency of occurrence at taps in homes supplied with potable mains water in the United States.
6. As expected, no positive correlations were observed between the non-fecal potential pathogens examined in this study (i.e., *L. pneumophila*, *M. avium*, *M. intracellulare*, *A. flavus*, *A. fumigatus*, and *A. niger*) and typical fecal indicator bacteria (i.e., TC, *E. coli*, and enterococci). Further, these potential pathogens were sometimes detected in treated rainwater samples via DNA-based methods even when no indicator bacteria were detected via MPN analyses. Thus, consistent with the rainwater literature, these data show that fecal indicator bacteria are insufficient to indicate non-fecal pathogens.
7. A general indicator of the biological quality of harvested rainwater is needed. A thorough assessment of the correlation between HPC and viable *L. pneumophila* concentrations should be undertaken to confirm their positive correlation in RWH systems.
8. In the non-chlorinated cisterns receiving water from Galvalume® roofs, DOC concentration was positively rank-correlated with HPC, *L. pneumophila* concentration, and *A. niger* concentration. The potential for limiting the occurrence/concentration of potential pathogens such as *L. pneumophila* and *A. niger* in harvested rainwater via removal of overhanging vegetation and regular

gutter/cistern cleaning should be evaluated; such activities might directly impact the introduction of pathogens to the cistern or indirectly impact pathogen concentration by affecting bioavailable DOC input to the cistern.

9. Sites with roof-wash or recirculating filters had significantly lower turbidity in the cisterns as compared to sites without them, but no significant difference in microbiological water quality was observed for those sites.

Succeeding task

DOC concentration was positively rank-correlated with HPC and *L. pneumophila* concentration, and pH was positively rank-correlated with *M. avium*. However, these correlations were calculated from cisterns that had many uncontrolled variables, so controlled bench-scale studies are needed. It is important to understand how physicochemical parameters in the cistern will impact microbiological water quality, particularly because rainwater harvesters can control pH, the presence of sediments, chlorine concentration, and possibly DOC concentration in their cisterns. Task 3 (Chapter 5) was designed to address this topic.

4. MICROBIOME OF HARVESTED RAINWATER BEFORE AND AFTER TREATMENT[3]

4.1. PROBLEM STATEMENT AND OBJECTIVES

The data from Task 1 (Chapter 3) showed that harvested rainwater treated by filtration and ultraviolet (UV) disinfection still contains a substantial quantity of unidentified heterotrophs (Figure 3.5a). However, very few non-culture-based microbiome analyses of harvested rainwater have been published (Chidamba & Korsten 2015, Cho & Jang 2014), and no studies exist on the treated harvested rainwater microbiome. Therefore, thorough interrogation of the microbiome of harvested rainwater before and after treatment is needed.

This chapter addresses **Task 2: Microbiome of harvested rainwater before and after treatment**. The objective of this task was to more fully understand the microbiome of harvested rainwater. For this purpose, DNA was extracted from rainwater samples at three full-scale residential rainwater harvesting (RWH) systems with treatment by filtration/UV light or by chlorination/filtration. Detailed taxonomic information was gathered using MiSeq® Illumina and Quantitative Insight into Microbial Ecology (QIIME).

The specific research questions addressed are as follows:

1. What is the microbiome of harvested rainwater?
2. Does site/treatment/season influence the microbiome of harvested rainwater?

[3] This chapter represents a summary of the manuscript that is being prepared for submission to the *Journal of Water and Health*.

4.2. MATERIALS AND METHODS

4.2.1. Sample selection

DNA was analyzed from three of the full-scale RWH systems described in Task 1 (Chapter 3). Site 1-Cl_2 (chlorinated cistern), site 2-UV (the site with the highest median heterotrophic plate counts [HPC] and fecal indicator bacteria concentrations among the Galvalume® roof sites both at the cistern and the cold, finished-water tap), and site 4-UV (site with the highest median mycobacteria concentrations at the cold, finished-water tap) were selected for analysis. Rainwater samples were vacuum-filtered through 0.22-µm polyethersulfone water filters (median of 1.0 L), and then DNA was extracted from the filter using a RapidWater® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). Twenty-six samples were sequenced (24 samples from 4 seasons × 3 sites × 2 sampling locations [cistern and cold, finished-water tap] + 2 additional samples collected from site 4-UV in the summer of 2012). Details of the sites, sampling locations, dates, and key features of the RWH systems are given in section 3.2.1.

4.2.2. MiSeq® Illumina and QIIME

For each DNA sample, amplicon of the V4–V5 region of the 16S rRNA gene was generated with primer set 515F (5'-Barcode-GTGYCAGCMGCCGCGGTA-3') and 909R (3'-TGARTTTMCTTAACYGCCCC-5') for Bacteria and Archaea (Hunicke-Smith 2015), and sequencing was performed with MiSeq® Illumina at the Institute for Cellular and Molecular Biology Genomic Sequencing and Analysis Facility at The University of Texas at Austin. QIIME v1.7.0 was used for sequence analysis (Caporaso et al. 2010), described as follows.

A mapping file was created to assign three characteristics (i.e., site, sampling location [cistern or tap], and season) to the 26 samples. The sequences were filtered for quality; then they were clustered into operational taxonomic units (OTUs) at 97% similarity according to the subsampling open-reference protocol using the October 2012 version of the GreenGenes (greengenes.secondgenome.com) reference database (Kueneman et al. 2014, DeSantis et al. 2006) and uclust (Edgar et al. 2011). Taxonomy was assigned by the Ribosomal Database Project (RDP) classifier (Wang et al. 2007). For α-diversity analysis, all the samples were normalized to an equal number of sequences because comparing two communities at different depths can be misleading; a random subsampling (rarefaction) was performed to obtain 10,800 sequence reads for each sample. The sample microbiomes were analyzed for α-diversity (observed OTUs) and β-diversity (weighted UniFrac distances). Compositions were classified at every taxon level to the genus level (i.e., phylum, class, order, family, and genus). Results from Task 1 (Chapter 3) were used to support the findings of the microbiome analyses.

4.2.3. Community calculations

Statistical analysis

Spearman's rank correlation was adopted to see if two parameters have a monotonic relationship (e.g., whether observed OTUs tend to increase or decrease when HPC increases). When a statistically significant correlation was found ($p < 0.05$), that correlation was visually inspected. Mann-Whitney tests, with a significance level of $\alpha = 0.05$, were used to determine if a metric for one group is significantly greater than or less than that for another group (e.g., weighted UniFrac distance among all site 1-Cl_2 cistern sample vs.

among all site 2-UV cistern samples). Statistical calculations were performed with MATLAB R2016a (MathWorks, Natick, MA) or with the Social Science Statistics web (http://www.socscistatistics.com).

Percent shared phylotype, colonizers, and transient OTUs

Percent shared phylotype between two samples (regardless of the relative abundance of each phylotype) was calculated as follows:

$$\%\ \text{shared phylotype} = \frac{\text{number of shared phylotypes between two samples}}{\text{number of observed phylotypes in the sample of interest}} \times 100$$

Colonizers were defined as microorganisms with OTUs (classified at the genus level) that were found in every sampling at a particular sampling location. When an OTU colonized only one particular cistern, this was termed a unique colonizer; when an OTU colonized more than one cistern, this was termed a shared colonizer. By contrast, when an OTU was not found in every sampling from a particular cistern, this was termed a transient OTU. The overall relative abundance of colonizers at a particular location was calculated as an average relative abundance across the sampling events. An example calculation for percent shared OTUs and colonizers is as follows:

> Three samples were collected from the same cistern in three different seasons (Samples 1-3). A, B, C, D, E, and F are OTU types, and the number in parentheses is the relative abundance of the corresponding OTU in that sample.
> - Sample 1: A (50%), B (30%), C (20%); number of observed OTUs is 3

- Sample 2: B (20%), C (70%), D (7%), E (2%), F (1%); number of observed OTUs is 5
- Sample 3: B (10%), D (20%), F (70%); number of observed OTUs is 3

The following observations can be made from these data:

1. Sample 1 shares 67% (2 out of 3; shares B and C) of phylotype with Sample 2, and the relative abundance of shared OTUs is 30% + 20% = 50%.
2. Sample 2 shares 40% (2 out of 5; shares B and C) of phylotype with Sample 1, and the relative abundance of shared OTUs is 20% + 70% = 90%.
3. The only colonizer in this cistern is OTU B. The overall relative abundance of the colonizer in this cistern is (30% + 20% + 10%)/3 = 20%, and the average relative abundance of all transient OTUs is 80%.

4.3. RESULTS

4.3.1. HPC, DNA concentration, and sequence number

The number of sequences from the 26 samples (including raw cistern water and finished tap water) ranged from 10,801 to 136,991, with an average of 80,713 sequences. Kuczynski et al. (2010) claimed that 1,000 sequences per sample is enough to describe overall patterns in bacterial community structure, so no sample was discarded. Despite low HPCs at the site 1-Cl_2 cistern and tap (< 100 colony-forming units [CFU]/mL), a high number of sequences were retrieved (Table 4.1). Rarefaction was performed at 10,800 sequences per sample prior to downstream analysis. Out of the 10,800 sequences from 26 samples, QIIME assigned the taxonomy of 9,748 to 10,714 sequences per sample (90.3%

- 99.2% of sequences), with an average of 10,254 sequences assigned per sample (94.9% of sequences).

Table 4.1: Median log heterotrophic plate count (HPC) (colony-forming unit [CFU]/mL), median rainwater filtrate volume (L), and median number of sequences from each sampling location

	Median log HPC (CFU/mL)	Median filtrate for DNA extraction (L)	Median number of sequences
Site 1-Cl_2 cistern	0.6	1.3	82,626
Site 1-Cl_2 tap	1.3	1.5	77,187
Site 2-UV cistern	6.3	1.3	85,331
Site 2-UV tap	4.1	1.3	79,628
Site 4-UV cistern	5.1	1.6	79,537
Site 4-UV tap	4.0	1.6	73,498

4.3.2. Observed OTUs

α-diversity (a measure of community richness or diversity within an individual sample) was calculated for each sample, ranging from 167 to 602 observed OTUs (Table 4.2). For non-disinfected rainwater cistern samples (sites 2-UV and 4-UV), observed OTUs were rank-correlated with HPC (Spearman's rho [r_s]= 0.76, p = 0.03) and with dissolved organic carbon (DOC) concentration (r_s = 0.81, p = 0.02). HPC and the DOC concentration were positively rank-correlated with community richness at the non-disinfected cisterns (Figure 4.1). It is possible that higher DOC concentrations lead to higher HPC and greater community richness.

Table 4.2: Observed operational taxonomic units (OTUs) from 10,800 sequences (α-diversity)

		Summer 2012	Fall 2012	Winter 2012	Spring 2013	Summer 2013
Site 1-Cl_2	Cistern	*	193	253	396	290
	Tap	*	244	314	302	425
Site 2-UV	Cistern	*	**602**	571	**439**	**463**
	Tap	*	579	**576**	427	415
Site 4-UV	Cistern	365	306	437	262	342
	Tap	198	167	308	234	205

*not sampled

Bold numbers represent the highest number in each season (site 2-UV samples always had the highest number of observed OTUs).

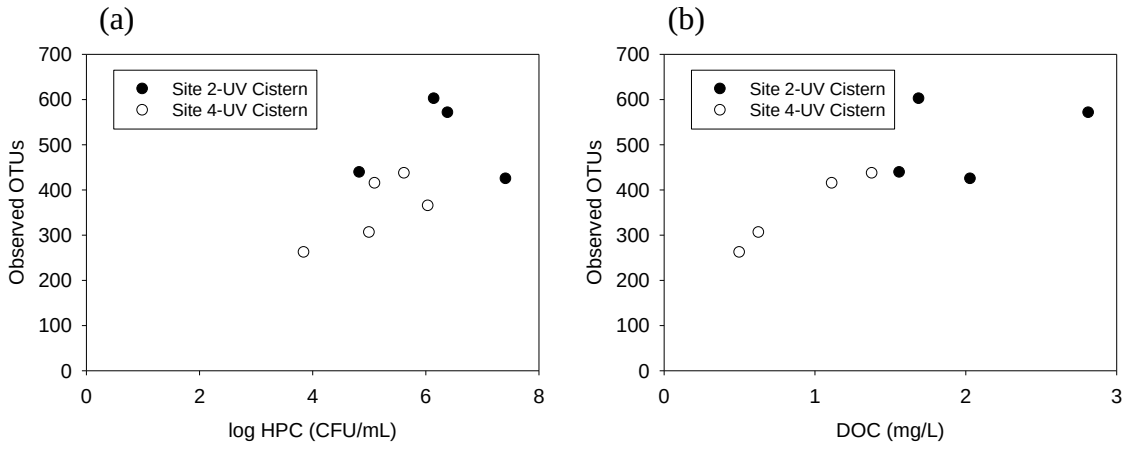

Figure 4.1: (a) Observed operational taxonomic units (OTUs) and heterotrophic plate count (HPC) of non-disinfected rainwater cistern samples (HPC plotted in log-scale), and (b) observed OTUs and dissolved organic carbon (DOC) concentration of non-disinfected rainwater cistern samples.

When observed OTUs were compared based on sampling location (cistern vs. tap), filtration/UV-treatment/distribution at sites 2-UV and 4-UV generally lowered the α-diversity (Table 4.2), as expected. Lin et al. (2016) also found that observed OTUs

decreased after UV treatment at two water reclamation plants. By contrast, at site 1-Cl_2 (where both cistern and tap samples contain chlorine), the tap samples generally had more observed OTUs as compared to cistern samples taken at the same time (3 out of 4 seasons; Table 4.2). This might indicate bacterial regrowth and/or the presence of biofilm in the pipes between the cistern and tap. There was a negative, but statistically insignificant, rank-correlation between residual chlorine and observed OTUs (r_s = -0.54, p = 0.17) (Figure 4.2a). The literature shows that temperature is a major factor affecting microbial community richness, where a positive correlation between temperature and community richness (e.g., seasonal fluctuation) has been observed (Pinto et al. 2014, Gilbert et al. 2012, Smit et al. 2001, Ferroni & Kaminski 1980). However, as shown in Figure 4.2b, temperature was not rank-correlated with observed OTUs at the non-disinfected cisterns (r_s = -0.18, p = 0.64).

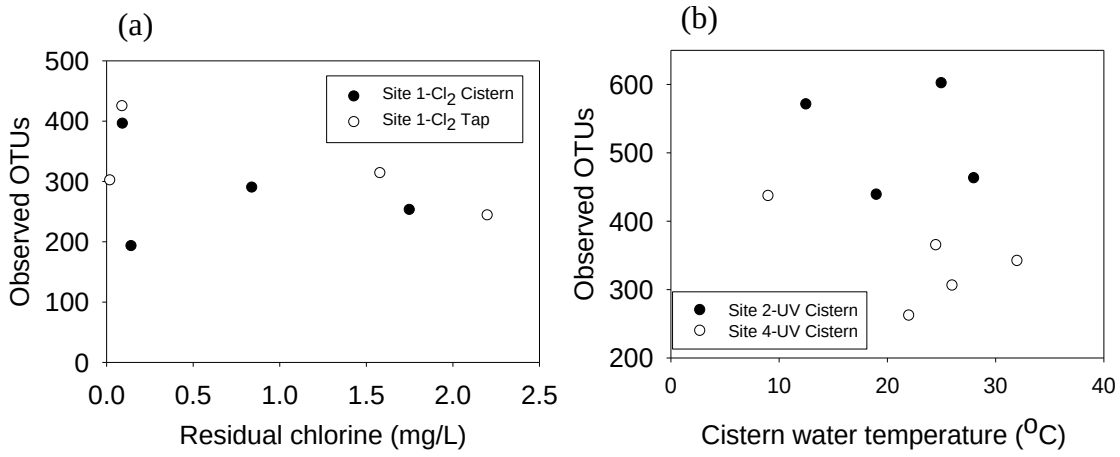

Figure 4.2: (a) Observed operational taxonomic units (OTUs) and residual chlorine of chlorinated samples, and (b) observed OTUs and water temperature of non-disinfected rainwater cistern samples.

4.3.3. Principal Coordinate Analysis (PCoA) (β-diversity)

β-diversity (a measure of diversity among samples) was calculated to determine which factors (e.g., site, sampling location, season) influence the harvested rainwater microbiome. To compare diversity among the samples, the weighted (i.e., accounting for the relative abundance of each taxon within the communities) UniFrac distance metric was calculated and then plotted in a two-dimensional Principal Coordinate Analysis (PCoA) plot (Lozupone & Knight 2005).

In the PCoA plots, the markers were shape-coded based on their season (Figure 4.3a) or site and sampling location (Figure 4.3b). Seasonal differences in the microbiome at each site were not obvious, but the data demonstrated that the most significant factor influencing the microbiome is site (note three distinctive clusters by site in Figure 4.3b). For instance, the two non-disinfected cisterns (sites 2-UV and 4-UV) had distinct cistern microbiomes even though the sites were geographically close (< 1 km apart). Although sites 2 and 4 had very similar systems (e.g., same cistern material and same roofing material), they had different roof orientations (Table 3.1), which could have caused differences in the quality of harvested rainwater captured at each site; Chang et al. (2004) found that roof orientation (facing southeast vs. northwest) impacted of roof runoff quality as measured by electrical conductivity and heavy metal concentrations. Additionally, the cisterns at sites 2-UV and 4-UV both seemed to maintain a very stable microbiome over the year-long sampling, where the markers for each cistern are clustered in Figure 4.3b.

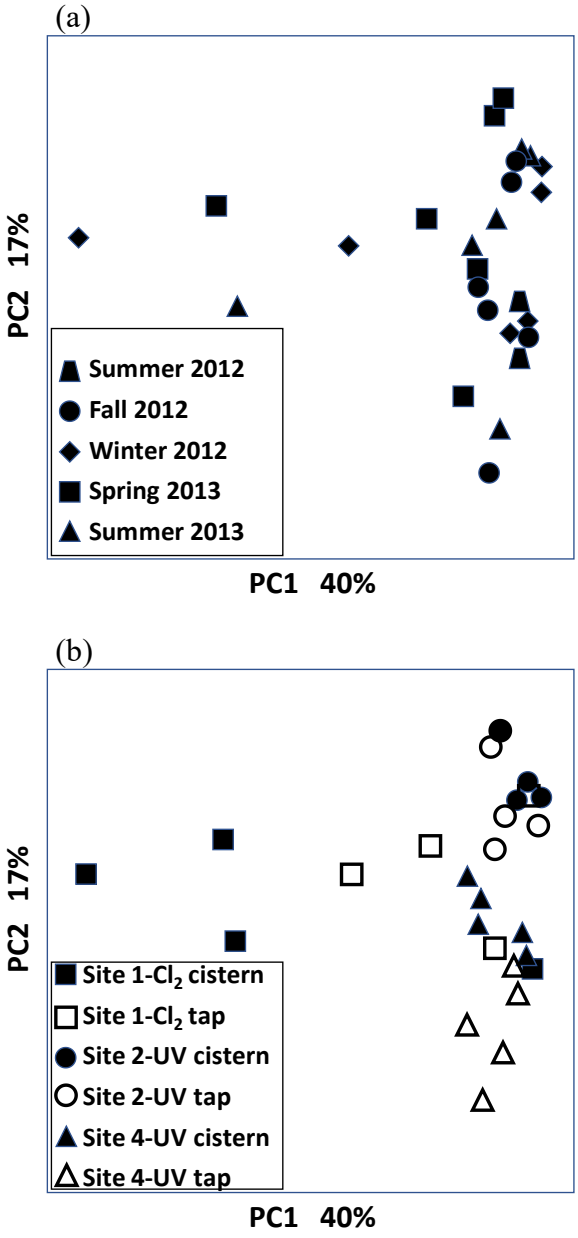

Figure 4.3: 2-D Principal Coordinate Analysis (PCoA) (β-diversity) plot of weighted UniFrac distance (a) by season and (b) by site and sampling location.

As shown in Figure 4.4, the weighted UniFrac distance among the site 1-Cl_2 cistern samples (Median [*Med*] = 0.61) was significantly greater than that for site 2-UV cistern samples (*Med* = 0.23), $Z = 2.80$, $p = 0.00$ and for site 4-UV cistern samples (*Med* = 0.26), $Z = 3.20$, $p = 0.00$. This means that the non-chlorinated cisterns maintained more stable microbiomes over the course of the sampling year than did the chlorinated cistern. The weighted Unifrac distance between the site 4-UV cistern and the site 4-UV tap (*Med* = 0.29) was significantly greater than that at site 2-UV (*Med* = 0.25), $Z = 2.18$, $p = 0.01$. Relatedly, Table 4.2 shows that observed OTUs decreased from the site 2-UV cistern to tap by 3% (median), while observed OTUs decreased by 40% (median) at site 4-UV. Taken together, these data suggest that site 4-UV treatment is more effective and/or that site 2-UV has more regrowth/biofilm between the cistern and the tap. The microbiome at site 1-Cl_2 (both at the cistern and tap) changed at each sampling, which suggests that batch chlorination substantially impacted the community structure. The weighted UniFrac distance between site 1-Cl_2 tap samples was significantly less than the distance between site 1-Cl_2 cistern samples, $Z = 2.64$, $p = 0.00$, which indicates less fluctuation in the microbiome composition at the tap (Figure 4.4). Biofilm formation between the site 1-Cl_2 cistern and tap, suggested earlier based on increases in the observed OTUs from the cistern to the tap (Table 4.2), might account for reduced variation in the microbiome at the site 1-Cl_2 tap as compared to the cistern.

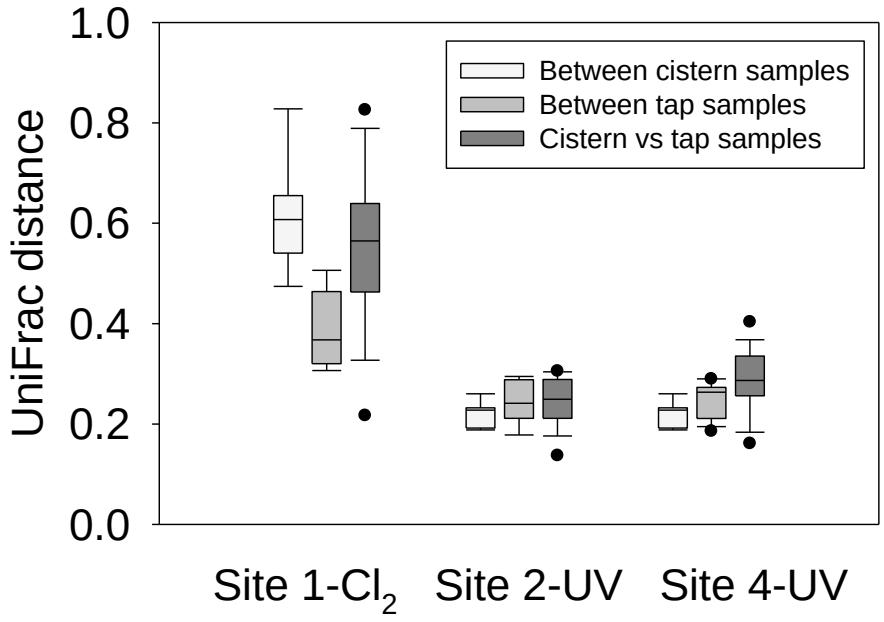

Figure 4.4: Weighted UniFrac distances within each site.

*Lines in boxes represent median values, vertical boxes represent 25th and 75th percentiles, error bars represent 10th and 90th percentiles, and dots represent 5th and 95th percentiles.

4.3.4. Microbiome composition

The relative abundance at the phylum level showed that harvested rainwater generally was dominated by Proteobacteria except at the site 1-Cl_2 cistern (Figure 4.5 - Figure 4.7). Proteobacteria is a very common phylum in natural water environments. For example, Proteobacteria was the dominant phylum found in harvested rainwater in South Africa (Chidamba & Korsten 2015) and in lakes and rivers in the Netherlands (Zwart et al. 2002), while comprising only 2.5% of the human fecal microbiome (Navas-Molina et al. 2013).

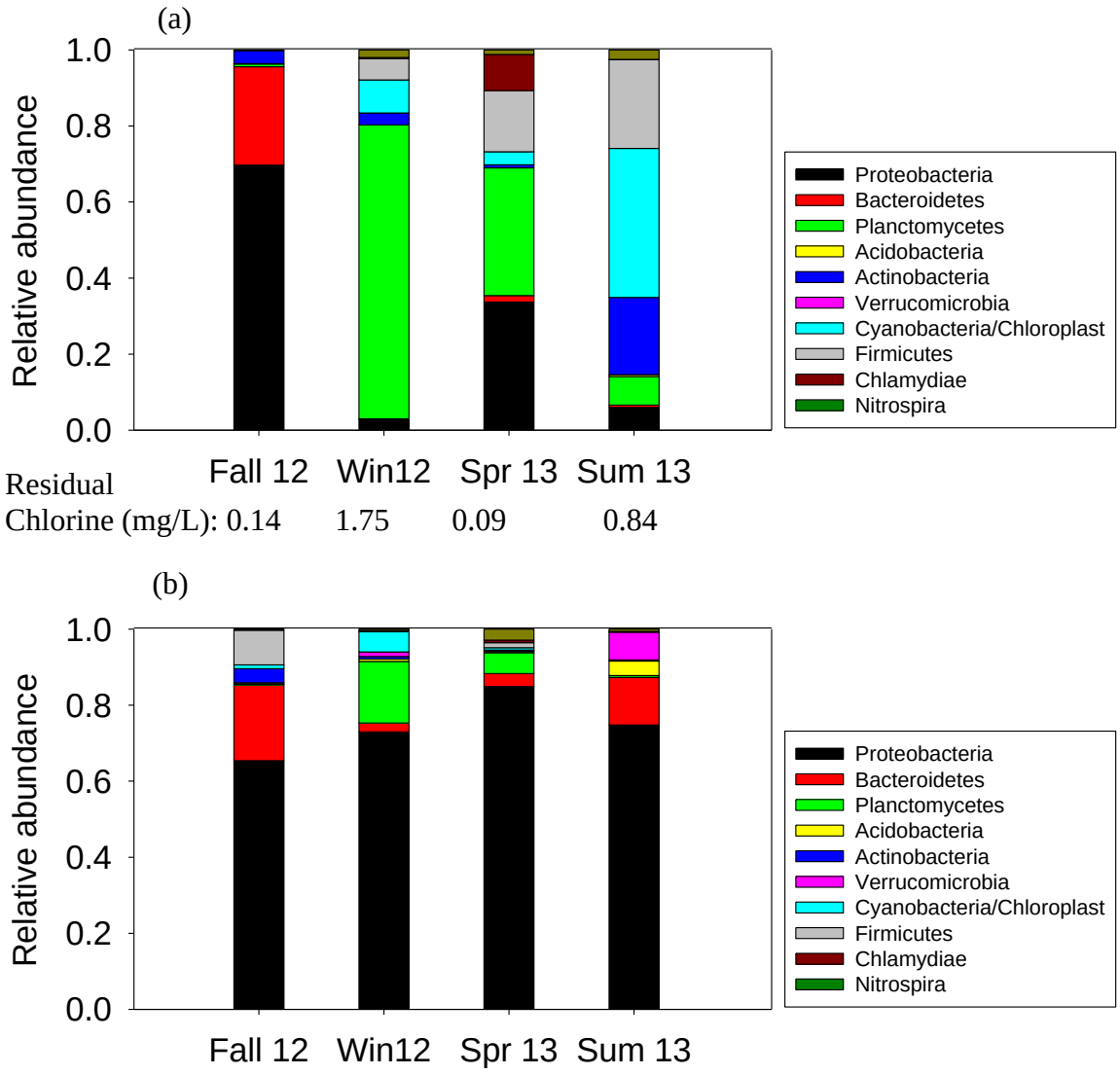

Figure 4.5: Seasonal phylogenetic distribution of operational taxonomic units (OTUs) at the phylum level at (a) site 1-Cl_2 cistern (residual chlorine concentration is noted below the figure) and (b) site 1-Cl_2 tap.

*Sum: Summer, Win: Winter, Spr: Spring.

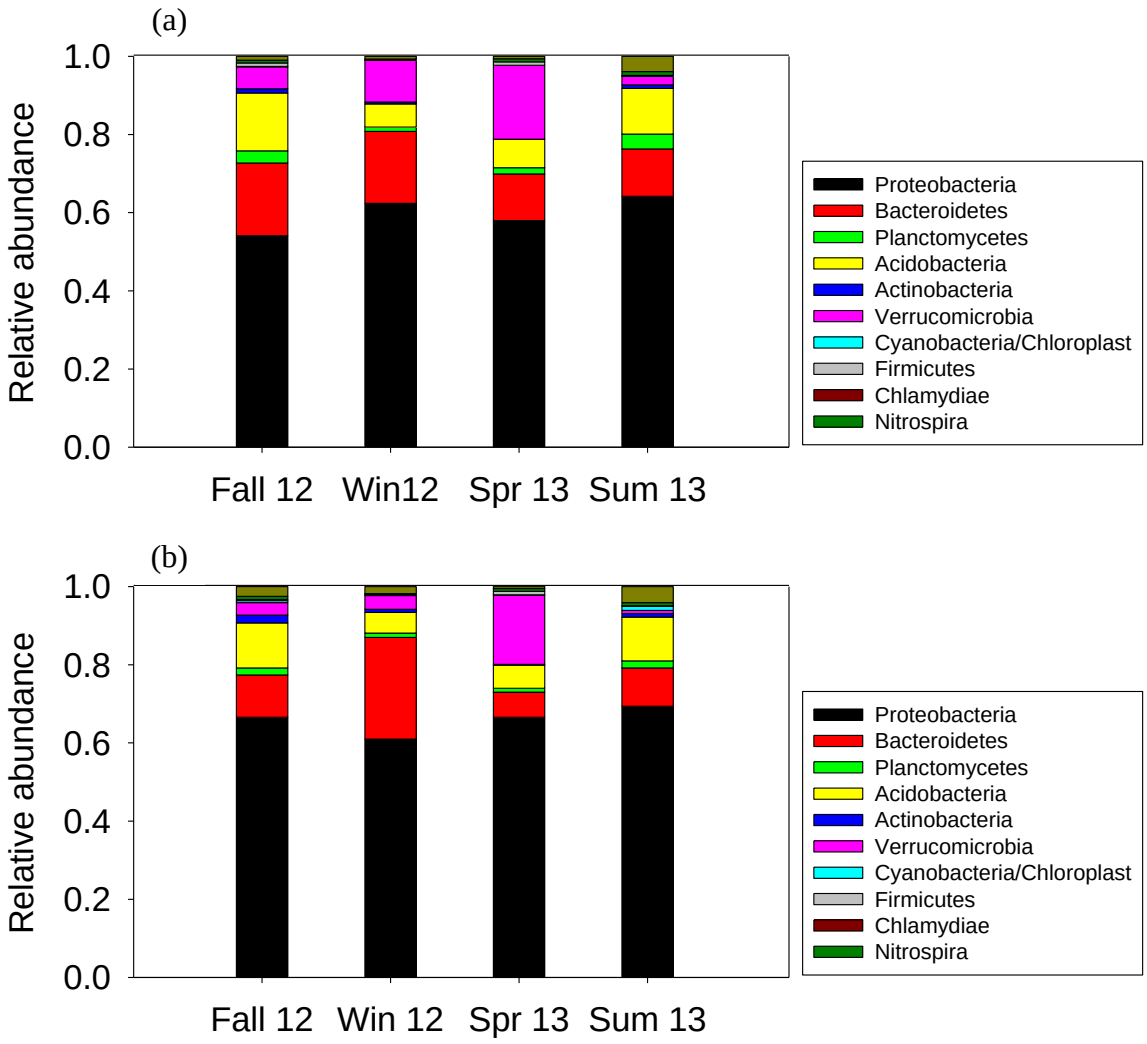

Figure 4.6: Seasonal phylogenetic distribution of operational taxonomic units (OTUs) at the phylum level at (a) site 2-UV cistern and (d) site 2-UV tap.

*Sum: Summer, Win: Winter, Spr: Spring.

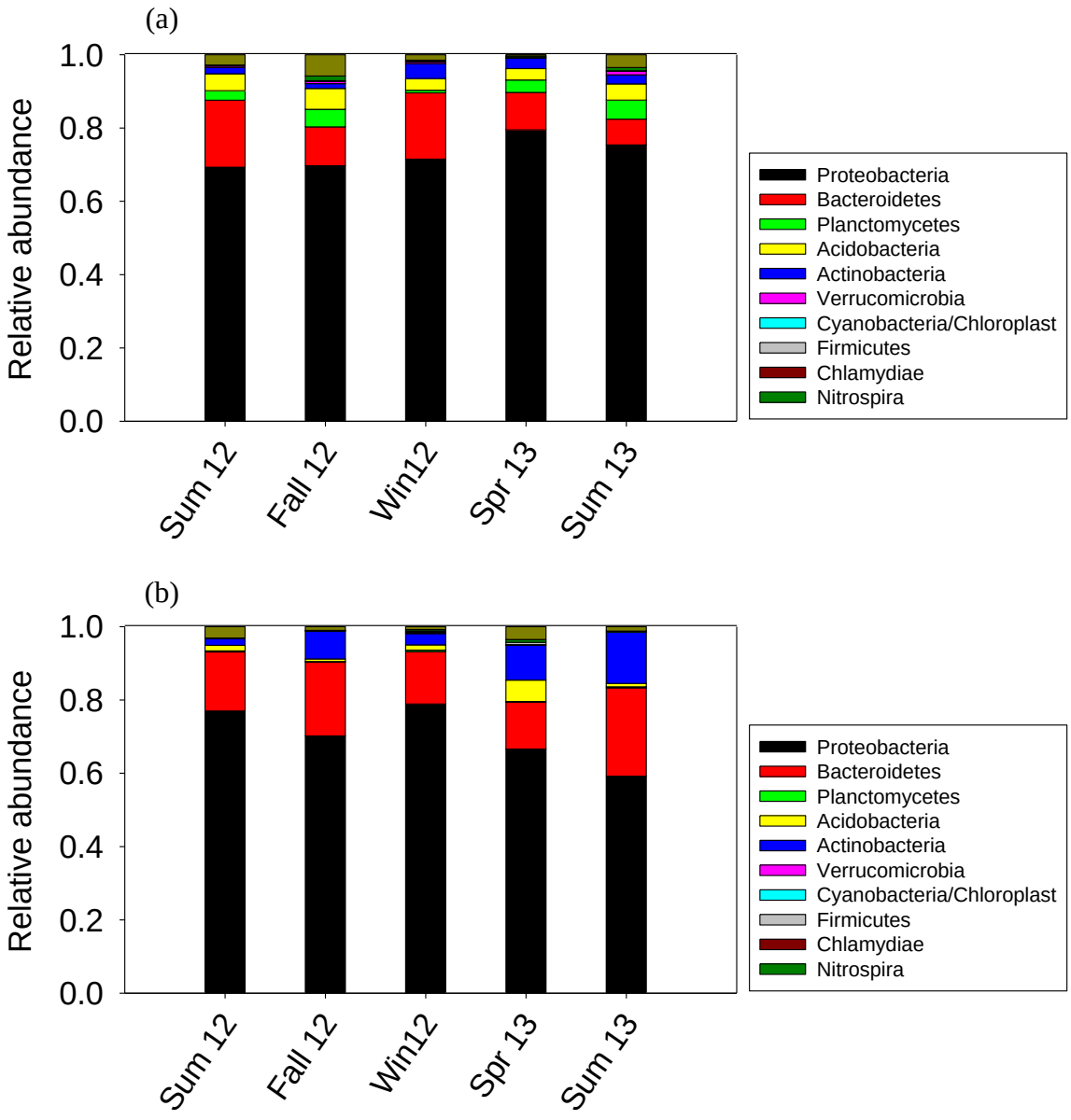

Figure 4.7: Seasonal phylogenetic distribution of operational taxonomic units (OTUs) at the phylum level at (a) site 4-UV cistern and (b) site 4-UV tap.

*Sum: Summer, Win: Winter, Spr: Spring.

Similar to what was concluded from the PCoA plot in Figure 4.3b, phylum-level analysis showed that the site 1-Cl_2 cistern had a unique microbiome at each sampling (where the dominant phylum changes every season in Figure 4.5a). The site 1-Cl_2 cistern generally had lower relative abundance of Proteobacteria and higher relative abundances of Actinobacteria and Firmicutes as compared to the other cisterns (Table 4.3, Figure 4.5 - Figure 4.7). Actinobacteria and Firmicutes are Gram-positive bacteria that are better able to tolerate disinfectants as compared to the more sensitive Gram-negative Proteobacteria (Meulemans 1987). At the genus level, *Alicyclobacillus* and *Pelotomaculum*, which are in the Firmicutes, were found in the site 1-Cl_2 cistern (median relative abundance of 6.3% and 0.4%, respectively), but they were not detected at other cisterns (data not shown).

Table 4.3: Median relative abundance (%) of Gram-positive phyla

	Site 1-Cl_2 Cistern	Site 1-Cl_2 Tap	Site 2-UV Cistern	Site 2-UV Tap	Site 4-UV Cistern	Site 4-UV Tap
Actinobacteria	3.3	0.5	0.7	0.9	2.5	7.5
Firmicutes	10.9	0.8	0.5	0.2	0.0	0.2

The non-disinfected cisterns (site 2-UV and site 4-UV) showed very little change in community composition across the seasons (Figure 4.6a, Figure 4.7a). This suggests that the non-chlorinated cistern microbiomes were resilient, with those cisterns maintaining/recovering their microbiomes even though an average of 70% of the cistern volume was refilled by an influx of fresh roof-harvested rainwater between the quarterly sampling events. At both UV sites, the relative abundance of Actinobacteria increased after filtration/UV-treatment/distribution, in 8 of 9 paired sampling sets (Figure 4.6, Figure 4.7, Table 4.3), which suggests that Actinobacteria have better survivability from UV

disinfection than do other phyla. Consistent with this, Bull (2011) noted that many studies found genera with UV-resistant species within Actinobacteria, such as *Brachybacterium*, *Geodermatophilus*, and *Rhodococcus*. Among the Firmicutes, two genera containing potential human pathogens (*Staphylococcus* and *Clostridium*) were detected more frequently in UV-treated water (4 times) than in the non-disinfected cisterns (1 time), which further suggests that Gram-positive bacteria might not be sufficiently controlled by a residential disinfection system.

4.3.5. Rank-correlations between *Legionella pneumophila* concentration and relative abundance of particular OTUs at genus-level classification

As shown in Table 4.4, Spearman's rank correlations showed that the relative abundance of five OTUs (classified at the genus level, but some only identified until the family level) were positively rank-correlated with the *L. pneumophila* concentrations found in Task 1 (Chapter 3). Although the OTU data cannot be mined to the species level, other studies have found a relationship between *L. pneumophila* and species that belonged to two of these five OTUs. For example, among the Acetobacteraceae family, *Craurococcus* spp. were found to be intra-amoebal like *L. pneumophila* (Thomas et al. 2006). Since evidence of amoeba has been found in rainwater cisterns (S. Bae and M J Kirisits, unpublished data), the presence of OTUs consistent with intra-amoebal bacteria are expected. Additionally, *Brevundimonas vesicularis* (in the Caulobacteraceae family) was found to support the growth of *L. pneumophila* in nutrient-poor environments (Koide et al. 2014).

Table 4.4: Rank-correlations between concentrations of *Legionella pneumophila* (from Task 1) and relative abundance of operational taxonomic units (OTUs) at the genus or family level in non-disinfected cisterns (9 samples)

Potential human pathogen	OTUs classified at the genus level	Spearman's rho (r_s)	p
L. pneumophila	Acetobacteraceae*	0.92	0.00
L. pneumophila	Sphingomonadaceae*	0.89	0.00
L. pneumophila	Chitinophagaceae*	0.77	0.03
L. pneumophila	*Polaromonas*	0.73	0.04
L. pneumophila	Caulobacteraceae*	0.70	0.05

Only statistically significant correlations ($p < 0.05$) are reported.

*Classified at the genus level, but only identified until the family level.

4.3.6. Percent shared phylotypes (microbiome similarity among the samples)

The percent shared phylotypes showed that the site 1-Cl_2 cistern has a substantially different microbial community every season; for example, only 15% of the phylotypes identified in the Winter 2012 sample also were found in the Fall 2012 sample (Table 4.5). However, the non-disinfected cisterns commonly had many shared phylotypes with the previous sampling (*Med* = 60%) as shown in Table 4.5.

Table 4.5: Percent shared phylotype with the previous sampling

	Fall 2012	Winter 2012	Spring 2013	Summer 2013
Site 1-Cl_2 cistern	-	15	36	41
Site 2-UV cistern	-	64	67	54
Site 4-UV cistern	72	49	60	43

Colonizer and transient OTUs were calculated using the OTU composition at the genus-level classification. As expected, the microbial communities of the non-disinfected cisterns had higher percentages of colonizers as compared to the site 1-Cl_2 cistern, and, in fact, the non-disinfected cisterns were dominated by colonizers (Figure 4.8).

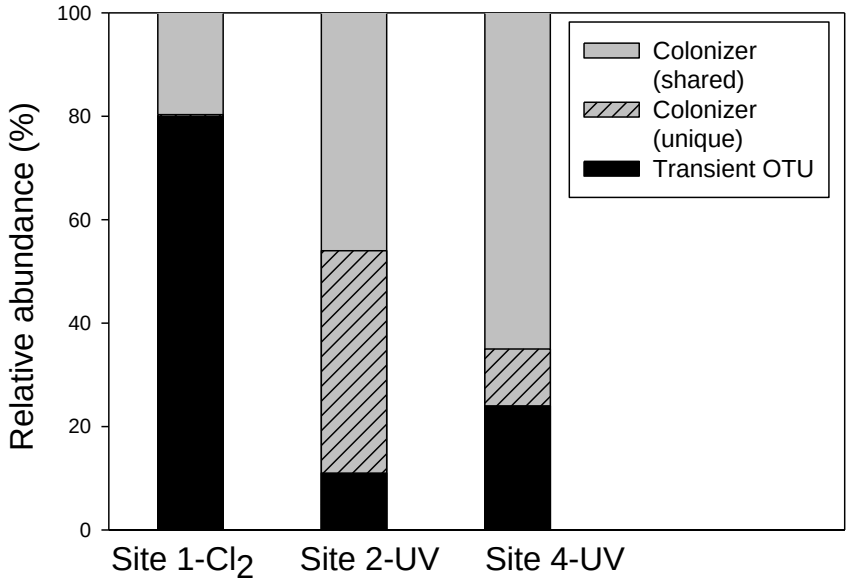

Figure 4.8: Overall relative abundance (%) of operational taxonomic units (OTUs) from colonizers (gray) and transients (black) in each cistern across the sampling events.

In Task 1 (Chapter 3), *L. pneumophila* was consistently detected at site 2-UV but was rarely detected in the other cisterns. This observation and high abundance of colonizers in the site 2-UV cistern (Figure 4.8) together suggest that some OTUs detected in the non-chlorinated cisterns did not originate from fresh rainfall between the sampling events but were cistern-aged bacteria. It is possible that sediment bacteria dominate the non-chlorinated cistern community, such that rainfall events and seasonal variation do not result

in substantial shifts to the cistern microbiome. The presence of such a persistent (indigenous) microbiome also might suggest that colonizers exert selective pressure on incoming bacterial species (intruders), such that intruders are outcompeted by cistern-aged bacteria. This type of bacterial community recovery from a disturbance has been reported previously in the natural environment during sewage decomposition in river (Korajkic et al. 2015). Understanding how the microbial quality of harvested rainwater changes over time after an influx of fresh rainfall is important.

4.3.7. Conclusion and succeeding task

In this chapter, the harvested rainwater microbiome was analyzed in three residential RWH systems where one system has chlorine disinfection followed by filtration and two systems have filtration followed by UV disinfection. The following conclusions were drawn:

1. Microbial community richness, as measured by observed OTUs, was rank-correlated with HPC and DOC concentration in the non-disinfected cisterns in a statistically significant fashion.
2. Although the three RWH sites are located within 1 km of one another, their cistern and cold, finished-water tap microbial communities were distinct among the sites.
3. The chlorinated cistern had a unique microbiome at each sampling event, but the microbiomes of the two non-disinfected cisterns were very stable over the period of a year, suggesting the resilience of the non-disinfected cistern microbiome. The non-disinfected cisterns also had high abundances of

colonizing bacteria, which were those detected at all sampling events at a particular site and location.

4. Samples taken after filtration/UV-treatment/distribution showed changes in the microbiome relative to that present in the associated cistern. Two genera containing potential human pathogens (*Staphylococcus* and *Clostridium*) were found more frequently in UV-treated rainwater than in the cistern.

Succeeding task

Non-chlorinated rainwater cistern microbiomes were very stable over the course of a year-long period, and 80% of OTUs in those cisterns were detected in every quarterly sampling at a particular cistern. Thus, it appears that many OTUs detected in the non-chlorinated cisterns were not entering in fresh rainfall between the sampling events but rather had colonized the systems. The presence of such a stable microbiome might suggest that colonizers exert selective pressure on incoming bacterial species (intruders) such that intruders are out-competed by colonizers, but temporal changes in the cistern microbiome after rainfall have not yet been reported. Understanding how rainwater microbial quality changes during storage in a cistern is very important because rainwater harvesters in some parts of the country depend on stored rainwater to last through extended dry periods. Task 3 (Chapter 5) was designed to see how the microbiome, in general, and the *L. pneumophila* concentration, in particular, changes over time in a cistern after an influx of fresh rainfall.

5. IMPACTS OF PHYSICOCHEMICAL CONDITIONS IN RAINWATER CISTERNS ON TEMPORAL CHANGES IN THE CISTERN MICROBIOME

5.1. PROBLEM STATEMENT AND OBJECTIVES

Chapter 3 (Task 1) data suggested that some physicochemical conditions impact microbiological rainwater quality in the harvested rainwater cistern. Rainwater harvesters can impact the physicochemical conditions in their cisterns by (a) adjusting pH (e.g., addition of sodium carbonate), (b) possibly reducing the dissolved organic carbon (DOC) concentration (e.g., preventing organic matter intrusion by pruning trees or cleaning leaf guards and gutters), (c) removing accumulated sediments, and (d) adding bleach into the cistern. Because the microbiome in a rainwater cistern impacts the finished-water microbiome (shown previously in Figure 4.4), it is important to understand how physicochemical factors in the cistern impact the cistern microbiome and the persistence of potential human pathogens such as *Legionella pneumophila*. *L. pneumophila* frequently is found in rainwater cisterns (Kobayashi et al. 2014, Ahmed et al. 2014). In 2006, a man was hospitalized with Legionnaires' disease in New Zealand, and the *L. pneumophila* serogroup 1 isolated from his rainwater harvesting (RWH) system was indistinguishable from the one isolated from his respiratory tract (Simmons et al. 2008). It is important to study if *L. pneumophila* exogenously delivered (e.g., by an influx of fresh rainfall) can survive and proliferate in rainwater cisterns.

As shown in Chapter 4 (Task 2), the microbiomes of closely located cisterns were distinct from one another, and the microbiomes of non-disinfected cisterns were stable over the period of one year. Since cistern rainwater can be held for extended periods during a dry season, observing shorter-term temporal changes in the cistern microbiome also is important.

To address these knowledge gaps, this chapter addresses **Task 3: Impacts of physicochemical conditions in rainwater cisterns on temporal changes in the cistern microbiome and on pathogen persistence**. The objective of this task was to use bench-scale cisterns with different physicochemical conditions (pH, DOC concentration, sediment presence, chlorination) to examine short-term temporal changes in the cistern bulk water microbiome and *L. pneumophila* concentration after an influx of fresh roof-harvested rainwater (Figure 5.1).

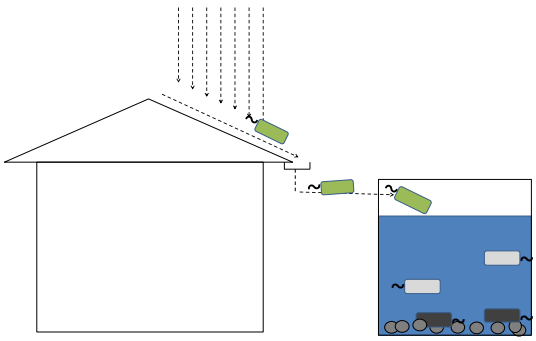

Figure 5.1: Rainwater cistern. The cistern contains microorganisms attached to the sediment (black), in the bulk cistern water (grey), and entering in fresh roof-harvested rainwater (green).

*Figure is not drawn to scale.

The specific research questions addressed were as follows:

1. Does the cistern microbiome proceed towards its pre-disturbance state after an influx of fresh roof-harvested rainwater?
2. Does pH, DOC concentration, presence of sediment, or chlorination in the cistern impact community richness, community recovery, and *L. pneumophila* persistence after an influx of fresh roof-harvested rainwater to the cistern?

5.2. MATERIALS AND METHODS

5.2.1. Bench-scale cistern preparation

Field site selection

The RWH system at the Austin Nature and Science Center (ANSC; Figure 5.2) was selected as the field site for this task. ANSC has a Galvalume® roof and a non-disinfected PVC cistern, which are features similar to many of the RWH systems used in Tasks 1 and 2 (Chapters 3 and 4). Cistern sediment, cistern-aged rainwater, and fresh roof-harvested rainwater were collected from this system. In particular, the ANSC cistern is less than 5 feet tall, so sediment could be collected using a hand-held bilge pump; additionally, the downspout could be easily diverted to a sampling container so fresh roof runoff was straightforward to collect (Figure 5.3).

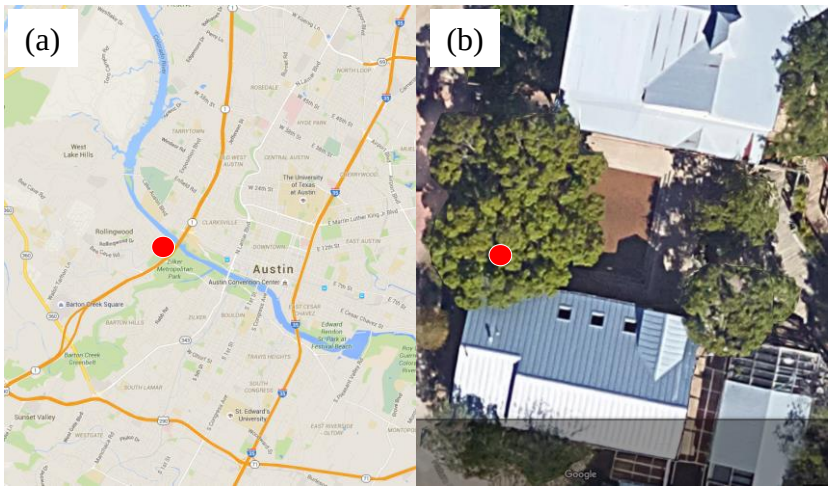

Figure 5.2: (a) The location of the Austin Nature and Science Center (red dot), (b) satellite image of the cistern location (red dot).

Figure 5.3: (a) Two rainwater collection points at the Austin Nature and Science Center (circled and numbered in the picture): [1] cistern-aged rainwater and sediment were collected to prepare bench-scale cisterns, [2] fresh roof runoff was collected directly from the building downspout during a rain event; (b) fresh roof-harvested rainwater was diverted from the building downspout via pipes into a polypropylene carboy.

Cistern-aged rainwater collection and bench-scale cistern preparation

Polyethylene carboys (60 L; ca. 8-inch water depth) were used as bench-scale cisterns (Figure 5.4a). In October 2016, sediment (shown in Figure 5.4b) was collected from the bottom of the ANSC rainwater cistern and then added to one bench-scale cistern to a depth of approximately 0.2 – 0.3 inch (coded as Cistern 7-Sed). On the same day, cistern-aged rainwater was collected from several inches below the water surface at the ANSC cistern. Cistern-aged rainwater (20 L) was transferred to each of eight bench-scale cisterns (coded as Cisterns 1 - 8) using a hand bilge pump, including the one bench-scale cistern to which sediment had already been added (Cistern 7-Sed). ANSC did not receive substantial rainfall for the antecedent 26 days, so the cistern water had been aged for about 4 weeks. The bench-scale cisterns were wrapped in aluminum foil and kept at room temperature (ca. 25 ^{o}C) without any disturbance for the next 13 days. The Task 3 timeline is summarized in Table 5.1.

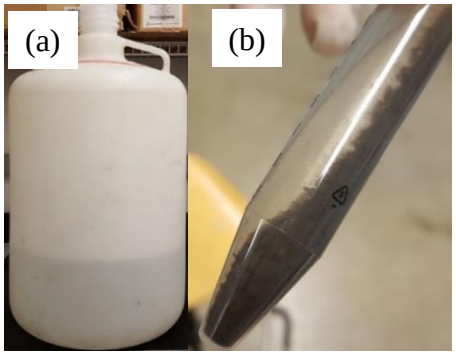

Figure 5.4: (a) Carboys that were used as bench-scale cisterns (foiled when the experiment started), and (b) sediment from the Austin Nature and Science Center cistern.

Table 5.1: Task 3 timeline

Day	-13	-4	-2	0^-	0	0^+	Through Day 28
Activity	Cistern-aged water and sediment collection from ANSC[a]	*Legionella pneumophila* cultivation on plate	Fresh roof-harvested rainwater collection from ANSC	(1) Cistern-aged rainwater, sediment, fresh roof-harvested rainwater sampling (2) *L. pneumophila* suspension preparation	Fresh roof-harvested rainwater and *L. pneumophila* addition to bench-scale cisterns	(1) Cistern physicochemical condition adjustment (2) Cistern sample collection for pH, turbidity, DOC[b], HPC[c], DNA analysis	Cistern sample collection for pH, turbidity, DOC, HPC analysis (twice per week), and DNA analysis (once per week)

[a]ANSC: Austin Nature and Science Center

[b]DOC: dissolved organic carbon

[c]HPC: heterotrophic plate count

5.2.2. Fresh roof-harvested rainwater and *L. pneumophila* spike

On day -4 (Table 5.1), an environmental *L. pneumophila* strain donated by Dr. Maura Donohue from the United States Environmental Protection Agency (USEPA) Cincinnati laboratory was transferred from a frozen stock to Buffered Charcoal Yeast Extract (BCYE) agar plates (BD, Franklin Lakes, NJ). The plate was placed in an anaerobic vessel with AnaeroPack™-Anaero Anaerobic Gas Generator (Mitsubishi™, Tokyo, Japan) and then incubated at 36 ^{o}C. The *L. pneumophila* strain was treated as biosafety level 2 as a precautionary measure.

On day -2, ANSC received rainfall (0.34 inch). This fresh roof-harvested rainwater (60 L) was captured from the RWH downspout at ANSC (Figure 5.3b) and stored at 4^{o}C.

On day 0^{-} (just before fresh roof-harvested rainwater and *L. pneumophila* addition), cistern-aged rainwater, sediment, and fresh roof-harvested rainwater were sampled for water quality analyses. *L. pneumophila* colonies were suspended in autoclaved deionized (DI) water (Figure 5.5). The *L. pneumophila* concentration was approximated by absorbance at 640 nm on a microplate reader, Synergy HT (BioTek, Winooski, VT). The relationship between absorbance at 640 nm and *L. pneumophila* concentration in colony-forming units (CFU)/mL was pre-established for various concentrations of *L. pneumophila* cells suspended in autoclaved DI water.

On day 0, fresh roof-harvested rainwater (5 L each) was poured into Cisterns 1 – 8 directly to ensure good mixing of water, which is the case at residential systems during a heavy rainfall. *L. pneumophila* (approximately 2.5×10^{6} CFU) was spiked to Cisterns 1 – 8, within 20 min of *L. pneumophila* stock preparation in DI water, for a final target concentration of approximately 10^{5} CFU/L. This concentration was chosen from Figure 3.7, where the *L. pneumophila* concentration at the site 2-UV cistern was between 10^{4} – 10^{5}

gene copy (gc)/L. To verify that *L. pneumophila* did not lose its viability as a result of the <20-min exposure to DI, the suspension was plated on BCYE agar plates on day 0^+, and the plates were counted on day 4. It was confirmed that the suspension contained the expected concentration of *L. pneumophila*, so viability had not been compromised.

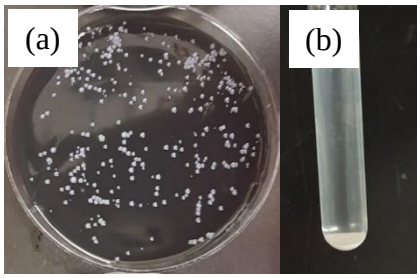

Figure 5.5: (a) *Legionella pneumophila* colonies on a Buffered Charcoal Yeast Extract (BCYE) agar plate, and (b) *L. pneumophila* suspension in autoclaved deionized water.

5.2.3. Cistern condition adjustment

The physicochemical condition of each bench-scale cistern (Table 5.2) was adjusted on day 0^+ (except for Cistern 7-Sed, where the sediment had been added on day -13), which is right after the fresh roof-harvested rainwater and *L. pneumophila* addition (Table 5.1). Cistern 1-Cont is the control, where only fresh roof-top harvested rainwater and *L. pneumophila* were added to the original 20 L of cistern-aged rainwater. Cistern 1-Cont had no adjustment of physicochemical conditions. In Cisterns 2, 3, and 4 (triplicates for pH-adjustment), the pH was adjusted from its ambient value (ca. pH 7.0) to pH 8 via sodium carbonate addition, which is practiced in some RWH systems to prevent corrosion of metal pipes. Sodium carbonate (0.8 g) was dissolved in 60 mL ultrapure water and then autoclaved. Then, 20 mL of sodium carbonate solution was added (100 μM final

concentration) to reach the target pH. The cisterns were gently shaken by hand for 5 seconds.

Table 5.2: Experimental setup of eight bench-scale cisterns

Cistern #	Simulated disturbance	Physicochemical conditions
1-Cont	Addition of fresh roof-top harvested rainwater (5 L) and *Legionella pneumophila* (ca. 2.5×10^6 colony-forming units) to 20 L of cistern-aged rainwater	No adjustment (pH ~7.0; no dissolved organic carbon [DOC] addition, no sediment addition)
2-pH1		pH adjustment (~ 8.0)
3-pH2		pH adjustment (~ 8.0)
4-pH3		pH adjustment (~ 8.0)
5-DOC1		DOC adjustment (organic tea; + 2 mg/L)
6-DOC2		DOC adjustment (organic tea; + 2 mg/L)
7-Sed		Sediment addition (0.2 – 0.3 inch)
8-Cl_2		Chlorination (~ 2 mg/L residual after 30 min)

The unadjusted DOC concentration in the cistern-aged rainwater was approximately 2 mg/L. The DOC concentration in a cistern could be affected by the presence of vegetation overhanging the catchment surface and the presence of organic debris in the gutters. To simulate this, the DOC concentration was increased by 2 mg/L in Cisterns 5 and 6 (duplicates for DOC-adjustment) by adding organic tea. The cisterns were gently shaken by hand for 5 seconds. To prepare the tea, leaves were collected near the ANSC cistern, soaked in ultrapure water for one day, and then filtered (0.45 μm). The DOC concentration of the organic tea was ca. 4,900 mg/L.

RWH systems can be equipped with gutter guards, roof-wash filters, and/or recirculating filters to reduce gross particulates entering the cisterns. With or without these features, sediments tend to accumulate in the cisterns over time. Sediments from the ANSC cistern were added to Cistern 7-Sed on day -13.

Cistern 8-Cl_2 simulates systems that perform batch chlorination, and 1.5 mL of bleach was injected directly to the cistern. The cistern was gently shaken by hand for 5 seconds. The target free chlorine residual was 2 mg/L after 30 min. The amount of bleach required to meet this target chlorine residual concentration was predetermined by using a 250-mL aliquot of cistern-aged rainwater.

One important difference between a real RWH cistern and the bench-scale cisterns is the seasonal temperature differences that occur in a real RWH (9 ^{o}C – 32 ^{o}C), while the bench-scale cisterns were kept in windowless room at constant temperature (ca. 25 ^{o}C). Another difference between a real RWH cistern and the bench-scale cisterns is the surface area to volume ratio (SA:V). In the cistern at ANSC, the SA:V increases from ca. 3 to ca. 9 m^{-1} while the cistern water decreases from full tank to 1/10 tank, but in the bench-scale cistern, SA:V increases from ca. 15 to ca. 16 m^{-1} while the rainwater volume decreases from 25 L to 20 L in the cistern.

5.2.4. Water quality analyses

Sampling events

As summarized in Table 5.1, water quality analyses and water filtration for DNA extractions (the filters were kept in a freezer) were performed on the cistern-aged water and fresh harvested rainwater on day 0^{-}. DNA also was extracted from the sediment on day 0^{-}. After the fresh roof-harvested rainwater and *L. pneumophila* spikes, temporal changes in the water quality and microbial communities of the cisterns were monitored at multiple time points during the 4-week experiment, as summarized in Table 5.1. DNA was extracted from the bulk water every week, and pH, DOC, heterotrophic plate count (HPC), and turbidity were measured twice per week. For the chlorinated cistern (8-Cl_2), the residual

chlorine concentration was measured 30 min after chlorine addition and on days 4 and 7. During the day 0^+ sampling (which occurred about an hour after chlorination), residual chlorine was quenched by sodium thiosulfate in the aliquots taken for HPC measurement and DNA extraction.

Physicochemical parameters and HPC

pH was measured with an Orion 720A pH/ISE/mV meter (Thermo Fisher Scientific, Waltham, MA). For DOC measurement, samples (40 mL) were filtered through a rinsed 0.45-µm nylon syringe filter (Fisher Scientific, Waltham, MA), acidified (sample pH < 2) with phosphoric acid, and stored in screw-capped borosilicate vials with Teflon-lined septa. DOC samples were preserved at 4 °C for up to two weeks and measured using a TOC-L (Shimadzu, Kyoto, Japan). HPC were conducted according to Standard Method 9215C (American Public Health Association (APHA) 2004). Samples were serially diluted in phosphate-buffered saline, plated on Difco™ R2A agar (Becton Dickinson, Franklin Lakes, NJ), and incubated at 28 °C for 7 days.

DNA extraction and **L. pneumophila** *quantification*

For DNA analyses, 1 L of water was vacuum-filtered through a 0.22-µm polyethersulfone water filter (MoBio Laboratories, Carlsbad, CA). Filters were kept in a freezer (ca. -20 °C) up to one month until DNA extraction, and DNA was extracted using a PowerWater® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). For sediment samples, 0.25 g of sediment sample was collected at day 0^-, and DNA was extracted using a PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA).

L. pneumophila (at the species level) concentrations were analyzed via quantitative, real-time polymerase chain reaction (qPCR). The primers and probe are summarized in Table 5.3. PCR reaction mixtures consisted of TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific, Waltham, MA), 0.4 μmol/L of each primer, 0.15 μmol/L of probe, and molecular-biology grade water. The ViiA™ 7 Real-Time PCR system (Applied Biosystems, Foster City, CA) at the Institute for Cellular and Molecular Biology core facility at The University of Texas at Austin was used. Briefly, the thermal cycler profile consisted of 50 ^{o}C (2 min), 95 ^{o}C (10 min), and 45 cycles of 95 ^{o}C (15 s) and 60 ^{o}C (1 min). Standard curves were constructed for every qPCR run. Accuracy, limit of detection (LOD), and precision for the analyses are reported in the Appendix.

Table 5.3: qPCR primers and probe targeting *Legionella pneumophila*

Primers and probe	**Sequence (5' → 3')**	**Reference**
Forward	TTGTCTTATAGCATTGGTGCCG	(Winchell & Benitez 2013)
Reverse	CCAATTGAGCGCCACTCATAG	
Probe	CGGAAGCAATGGCTAAAGGCATGCA	

5.2.5. Microbiome analyses

DNA concentration and purity were tested with a Nanodrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and DNA concentration was normalized before MiSeq® sequencing (Illumina, San Diego, CA). The 16S rRNA gene amplicon (V4) was generated with primer set 515F (5'-Barcode-GTGYCAGCMGCCGCGGTA-3') and 806R (3'-TAATCTWTGGGVHCATCAGG-5') targeting Bacteria (Wang & Qian 2009) and then sequenced at the Institute for Cellular and

Molecular Biology Genomic Sequencing and Analysis Facility at The University of Texas at Austin. The sequencing data were processed with Quantitative Insight into Microbial Ecology (QIIME) v1.7.0 to obtain taxonomic and phylogenetic information about the microbiomes (Caporaso et al. 2010). Details about the QIIME process is given in section 4.2.2. The following topics were analyzed: (1) temporal changes in the microbiome of cistern water (e.g., phylum- and genus-level distribution), (2) α-diversity, with an observed operational taxonomic units (OTUs) metric, to see community richness, and (3) β-diversity, with the weighted UniFrac metric, among the samples (i.e., samples with different physicochemical condition and different water age) to examine microbiome resilience.

5.2.6. Data analysis

Spearman's rank correlation was adopted to determine if two parameters have a monotonic relationship (e.g., whether observed OTUs tend to increase or decrease when HPC increases). One-tailed Mann-Whitney tests, with a significance level of $\alpha = 0.05$, were used to identify statistically significant differences between selected groups of samples.

5.3. RESULTS AND FUTURE WORK

5.3.1. Physicochemical water quality

Table 5.4 shows the physicochemical parameters of cistern-aged rainwater and fresh roof-harvested rainwater. The physicochemical conditions of cistern-aged rainwater from the ANSC cistern are in the range observed for the residential cisterns in Task 1 (Chapter 3).

Table 5.4: Physicochemical conditions of cistern-aged rainwater and fresh roof-harvested rainwater collected at the Austin Nature and Science Center

	pH	Turbidity (NTU[a])	DOC[b] (mg/L)	log HPC[c] (CFU[d]/mL)	*Legionella pneumophila* (genomic targets/L)
Cistern-aged rainwater	7.0	0.9	2.1	7.7	$10^{4.7}$
Fresh roof-harvested rainwater	7.2	0.9	5.2	6.8	$< 10^{2.7}$ (limit of detection)

[a]nephelometric turbidity unit
[b]dissolved organic carbon
[c]heterotrophic plate counts
[d]colony-forming unit

From day 0^+ to day 28, Cisterns 1 and 5-8 (without pH adjustment) maintained near-neutral conditions (Figure 5.6a). All the samples had pH between 6.8 and 7.1, except for Cistern 8-Cl_2 at day 0^+ (pH 7.3). For Cisterns 2-4 (-pH), to which sodium carbonate had been added, the pH decreased from about 8 to 7.4 by day 7 and maintained at that level until day 28. Turbidity from all the cisterns decreased slowly over time due to sedimentation in the undisturbed water columns (Figure 5.6b). Cistern 7-Sed had higher turbidity at day 0^+ compared to other cisterns, possibly due to the disturbance of the sediments by addition of the fresh roof-harvested rainwater, but the turbidity decreased to the level of the other cisterns by day 4. When the turbidities were compared over time, turbidity dropped significantly from day 0^+ (Median [*Med*] = 0.5 nephelometric turbidity units [NTU]) to day 4 (*Med* = 0.3 NTU) day, $Z = 3.31$, $p = 0.00$, and then again from day 14 (*Med* = 0.3 NTU) to day 18 (*Med* = 0.2 NTU), $Z = 1.73$, $p = 0.04$.

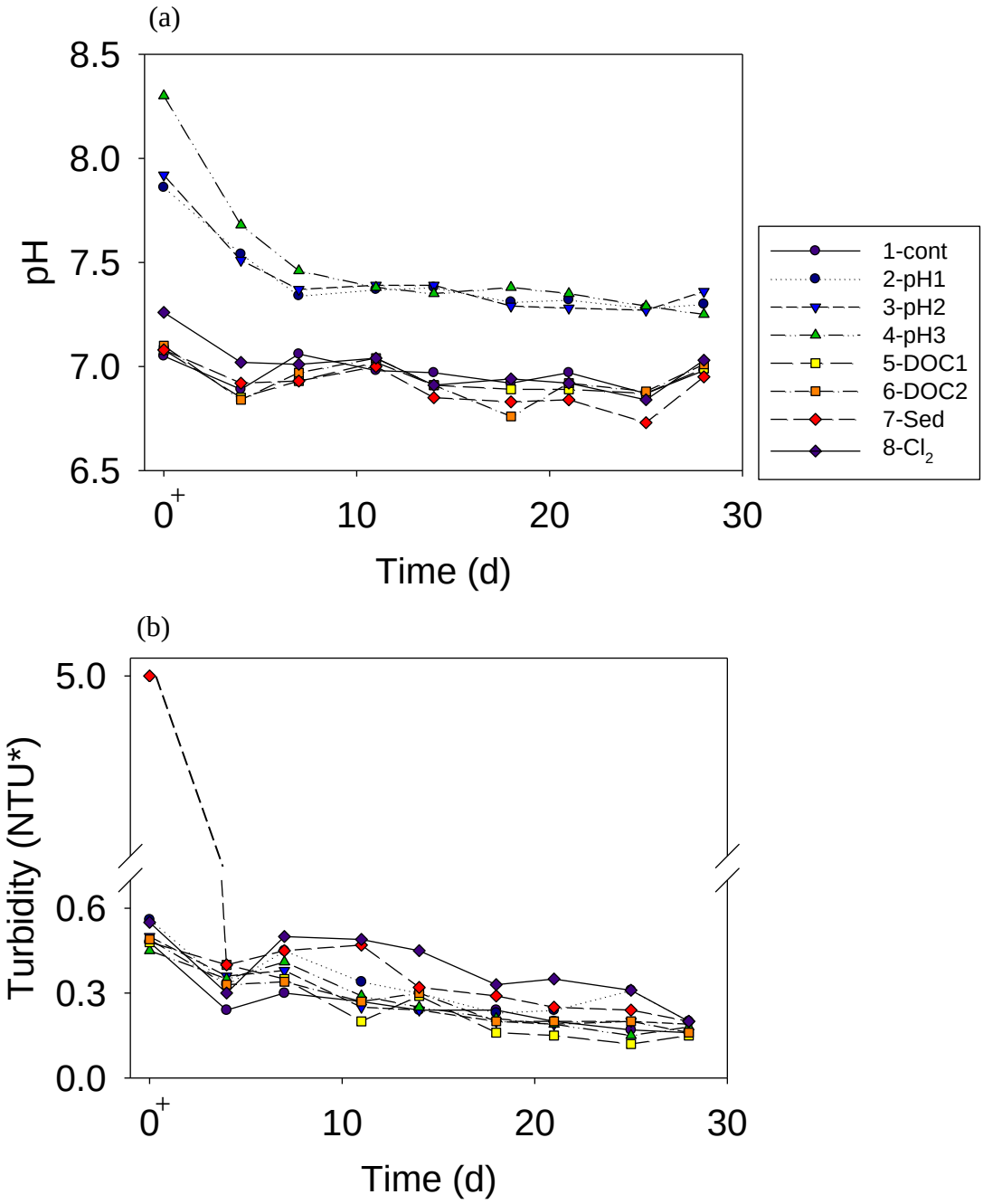

Figure 5.6: (a) pH and (b) turbidity in the bench-scale cisterns over time.

*NTU: nephelometric turbidity unit

The DOC concentrations of cistern-aged and fresh roof-harvested rainwater were 2.1 and 5.2 mg/L, respectively. The DOC concentration at day 0^+ (after the addition of 5 L fresh roof-harvested rainwater to the 20 L of cistern-aged rainwater) ranged from 2.5 mg/L to 2.8 mg/L in Cisterns 1-4 and 7 (Figure 5.7a). Cisterns 5 and 6 (-DOC) had DOC concentrations of 4.4 and 4.9 mg/L at day 0^+, where the difference between the two cisterns might due to insufficient mixing of the organic tea prior to addition to the cisterns. In Cisterns 1-4 and 7, the DOC concentrations generally decreased over time until day 17-21 and then remained relatively constant; this suggests that fresh roof-harvested rainwater delivers biodegradable organic carbon to the cistern and/or that DOC sorbed to surfaces or particles in the cisterns. For Cisterns 5 and 6 (-DOC), the exogenously added DOC was removed before day 11, suggesting that the organic tea was highly biodegradable and/or sorbable. From day 0^+ to day 7, Cisterns 5 and 6 (-DOC) had significantly higher DOC concentrations (*Med* = 3.2 mg/L) than did Cisterns 1-4 and 7 (*Med* = 2.5 mg/L), $Z = 2.1$, $p = 0.02$. From day 11 to day 28, the difference in DOC concentration between Cisterns 5 and 6 (*Med* = 2.3 mg/L) and the other cisterns (*Med* = 2.2 mg/L) became insignificant, $Z = 0.71$, $p = 0.24$. The DOC concentration in Cistern 8-Cl_2 increased from day 0^+ to day 7, and then it slowly went down to 2 mg/L. When all the data from day 0^+ to day 28 were compared, Cistern 8-Cl_2 had significantly higher DOC (*Med* = 2.9 mg/L) than did Cisterns 1-4 and 7 (*Med* = 2.4 mg/L), $Z = 2.55$, $p = 0.01$. It is possible that the microorganisms inactivated by chlorination contributed to the temporary increase in DOC concentration (e.g., through cell lysis); then, as the microorganisms regrew when the chlorine residual dissipated, the DOC concentration decreased due to microbial consumption.

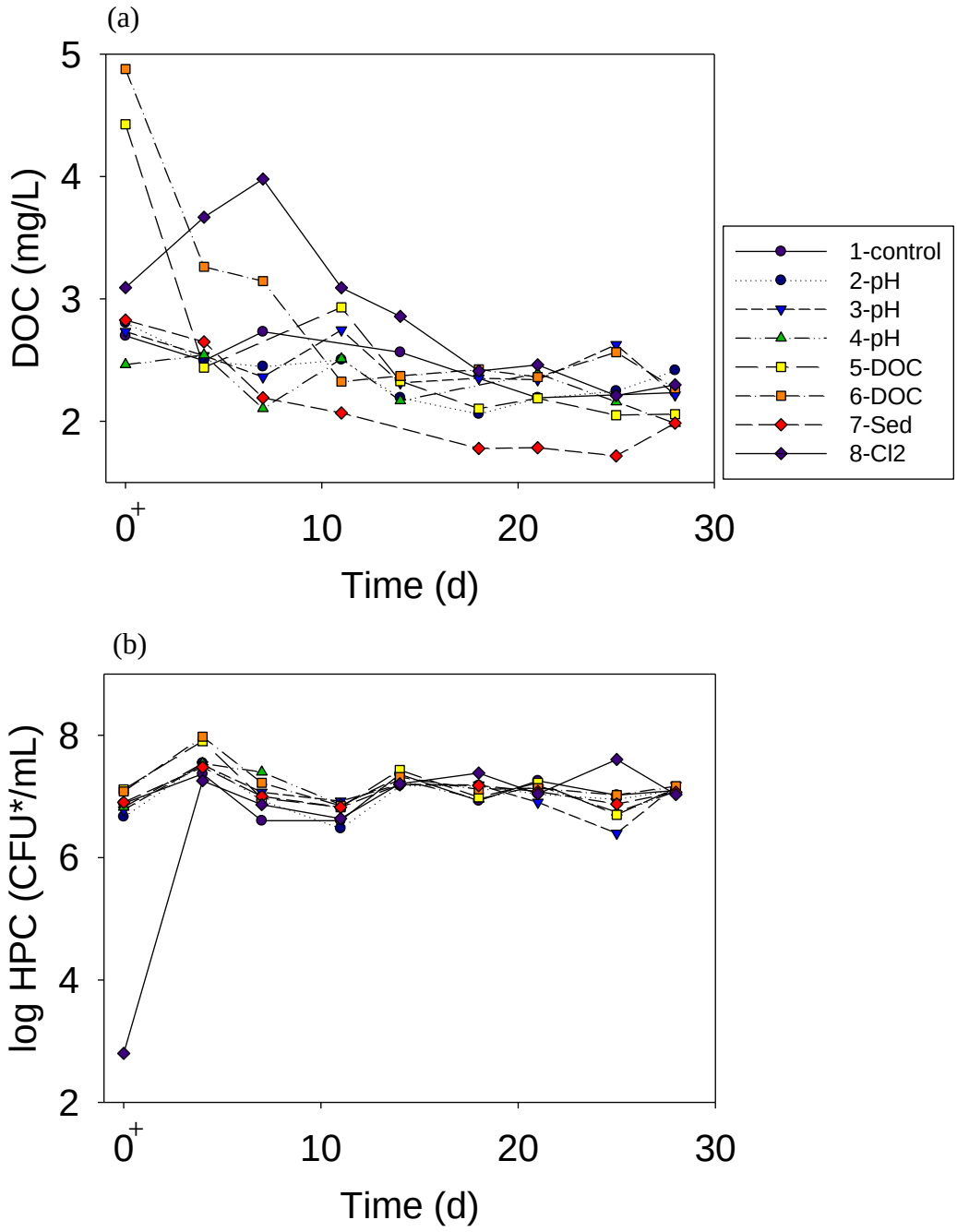

Figure 5.7: (a) Dissolved organic carbon (DOC) concentration and (b) Heterotrophic plate count (HPC) in the bench-scale cisterns over time.

*CFU: colony-forming unit

At Cistern 8-Cl_2, the log HPC was less than 3 on day 0^+ (due to one hour of chlorine disinfection at that sampling event), but HPC recovered by day 4. On day 4, the residual chlorine concentration was lower than the LOD of 0.05mg/L). From day 4 to day 28, the log HPC at Cistern 8-Cl_2 (*Med* = 7.0 in CFU/mL) was statistically indistinguishable from that at the other cisterns (*Med* = 7.1 in CFU/mL), $Z = 0.37$, $p = 0.36$. Therefore, batch chlorination caused only a temporary decline in HPC in the cistern.

The effect of organic tea addition at Cisterns 5 and 6 (-DOC) on HPC also was temporary. The HPC at Cisterns 5 and 6 (-DOC) were 0.5-0.6 log higher than at other cisterns on day 4 (Figure 5.7b). However, from day 7 to day 28, the log HPC at Cisterns 5 and 6 (*Med* = 7.1 in CFU/mL) were statistically indistinguishable from that at other cisterns (*Med* = 7.1 in CFU/mL), $Z = 0.77$, $p = 0.22$.

5.3.2. *L. pneumophila* concentration

In cistern-aged rainwater (day 0^-), the *L. pneumophila* concentration was $10^{4.7}$ gc/L, and the *L. pneumophila* concentration in fresh roof-harvested rainwater was lower than the LOD of $10^{2.7}$ gc/L (Table 5.4). After the *L. pneumophila* spike to the bench-scale cisterns on day 0^+, the *L. pneumophila* concentrations increased to ca. 10^6 gc/L, but slowly returned to a level close to the initial concentration (Figure 5.8). It is possible that *L. pneumophila* that was cultured on a high-nutrient BCYE agar plate (1) could not utilize nutrients in oligotrophic environment of harvested rainwater and/or (2) could not establish symbiotic relationship with other microorganisms. In Cistern 8-Cl_2, *L. pneumophila* was not detected over the entire 28-d period, even though HPC showed a full recovery 4 days after chlorination. This shows that chlorination is an effective way to control *L. pneumophila* in the cistern; however, as shown in the full-scale studies in Figure 3.7, controlling *L.*

pneumophila in the cistern does not necessarily prevent it from colonizing the downstream distribution system. Even though none of the bench-scale cisterns supported net growth of *L. pneumophila*, Cisterns 2-4 (-pH) had higher *L. pneumophila* concentrations than any other cisterns on day 7 and showed a lower rate of decline from day 0^+ to day 7 (Figure 5.8). Then, the *L. pneumophila* concentration dropped to a level close to the initial concentration by day 28. pH dropped from ca. 8.0 to 7.4 between day 0^+ and day 7, and it maintained around 7.4 thereafter (Figure 5.6a). It is possible that the higher pH in those cisterns resulted in slower decay of *L. pneumophila* before day 7. Wadowsky et al. (1985) cultured *L. pneumophila* in pH-adjusted tap water (pH ranged from 5.0 to 10.5) and counted viable cells until day 28. The data showed multiplication occurred at pH between 5.5 and 9.5, and *L. pneumophila* concentration was highest at pH 8.5 on day 7. Task 2 (Chapter 4) suggested several correlations between *L. pneumophila* and OTUs such as *Limnohabitans* spp. and *Polaromonas* spp. in the full-scale cisterns. No such correlations were found in the bench-scale cisterns, possibly because *L. pneumophila* had been exogenously spiked into the systems at time 0^+.

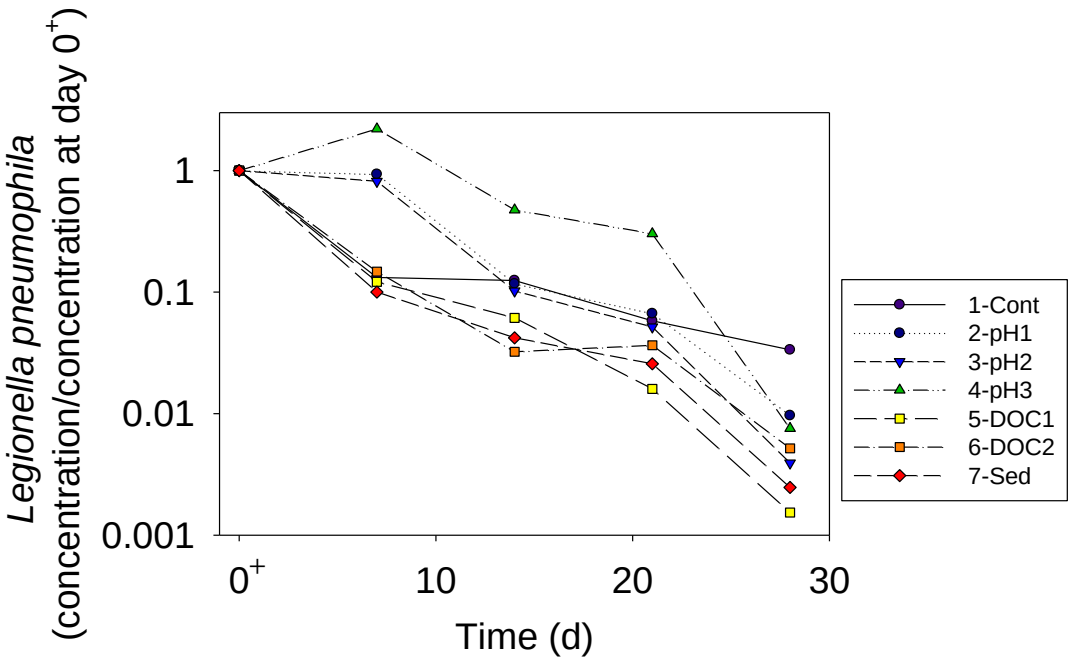

Figure 5.8: *Legionella pneumophila* concentrations (compared to its concentration at day 0^+ in each cistern) over time. *L. pneumophila* was not detected in Cistern 8-Cl_2.

5.3.3. Observed OTUs

Illumina® and QIIME analysis were performed on 43 samples: 40 bulk water samples (8 cisterns at five time points), the cistern-aged rainwater at time 0^-, the sediment at time 0^-, and the fresh roof-harvested rainwater sample at time 0^-. The metric "observed OTUs" was chosen to compute α-diversity. To calculate diversity at the same sequencing depth, rarefaction at 1075 sequences was performed. Five samples with fewer than 1075 sequences were discarded for the observed OTU calculation.

After rarefication, fresh roof-harvested rainwater had 644 unique OTUs, which is the 7th highest among all the samples; this indicates that fresh roof-harvested rainwater delivers a diverse microbiome to the cistern. After an influx of fresh roof-harvested rainwater (day 0^{+}), the number of observed OTUs increased over time in all the cisterns (Figure 5.9). When all the cisterns were compared, the number of observed OTUs at any time point was significantly higher than it was 14 days earlier; for example, the number of observed OTUs at day 28 (*Med* = 647) was significantly higher than that at day 14 (*Med* = 597), $Z = 1.79$, $p = 0.04$. The chosen modulations in cistern physicochemical condition (increasing the pH, adding DOC, adding sediments) did not substantially affect the observed OTUs.

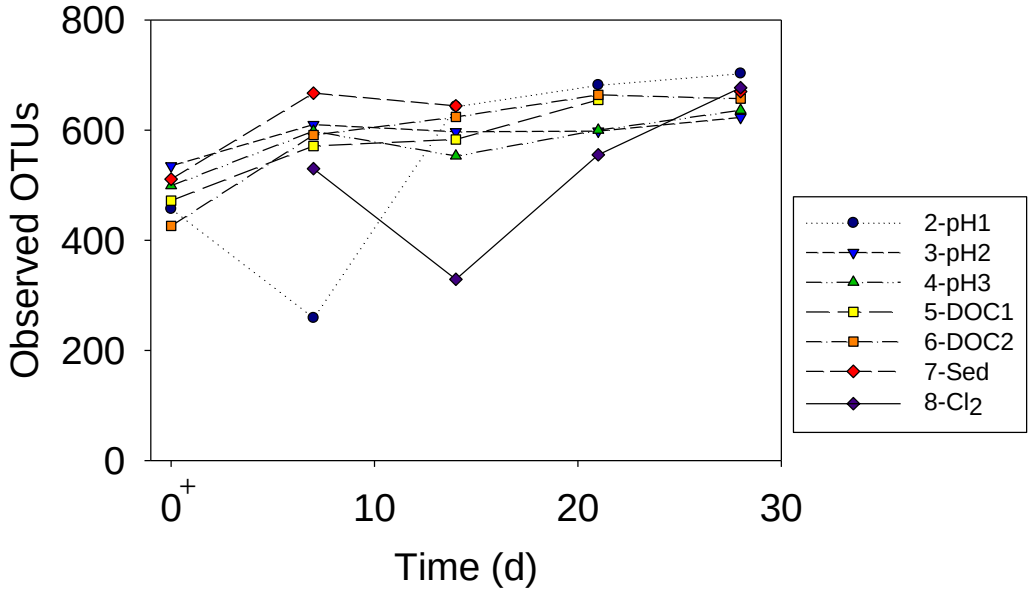

Figure 5.9: Observed operational taxonomic units (OTUs) in the bench-scale cisterns over time.

*Samples with lower than 1075 sequences were not included in this plot (e.g., Cistern 8-Cl_2 at day 0^{+}).
*Cistern 1-Cont is not shown because those samples are being resequenced.

Observed OTUs and HPC in the full-scale cisterns (Figure 4.1a) showed a positive rank-correlation. Similarly, observed OTUs and HPC in the bench-scale cisterns (Figure 5.10) showed a positive rank-correlation (Spearman's rho [r_s] = 0.39 p = 0.01).

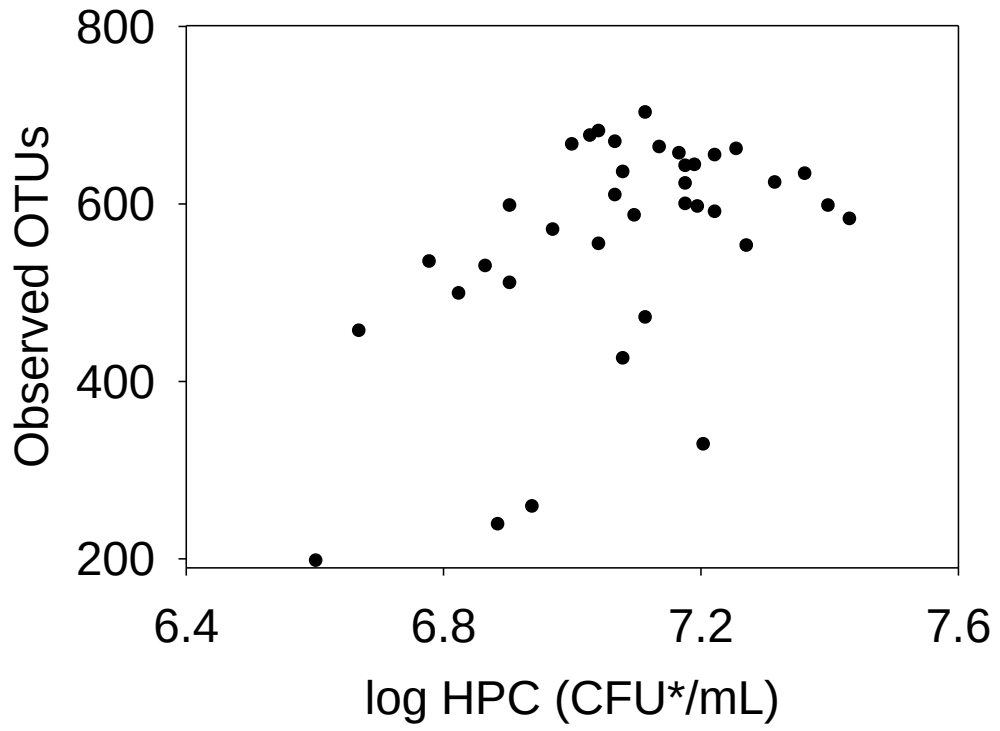

Figure 5.10: Observed operational taxonomic units (OTUs) and heterotrophic plate count (HPC), which was plotted in log-scale, in the bench-scale cisterns.

*CFU: colony-forming unit

5.3.4. Weighted UniFrac distance (β-diversity)

From cistern-aged rainwater to each cistern and from fresh roof-harvested rainwater to each cistern

For β-diversity calculations (i.e., diversity among the samples), the weighted UniFrac metric was used (Lozupone & Knight 2005). The weighted UniFrac distance from the original microbial community in the cistern-aged rainwater (day 0^-) to each sample showed that the cistern microbiomes were proceeding toward the pre-disturbance state (day 0^-) over time (Figure 5.11a).

The weighted Unifrac distance between the cistern-aged rainwater and fresh roof-harvested rainwater was 0.36. After an influx of fresh roof-harvested rainwater to the cistern-aged rainwater in each bench-scale cistern, the average weighted Unifrac distance between the community of each cistern and the community of fresh roof-harvested rainwater became 0.20 at day 0^+ (Figure 5.11b) demonstrating that the cistern bacterial communities moved closer to that of fresh roof-harvested rainwater, as expected. Also as expected, the cistern communities shifted farther from that of fresh roof-harvested rainwater over time (Figure 5.11b).

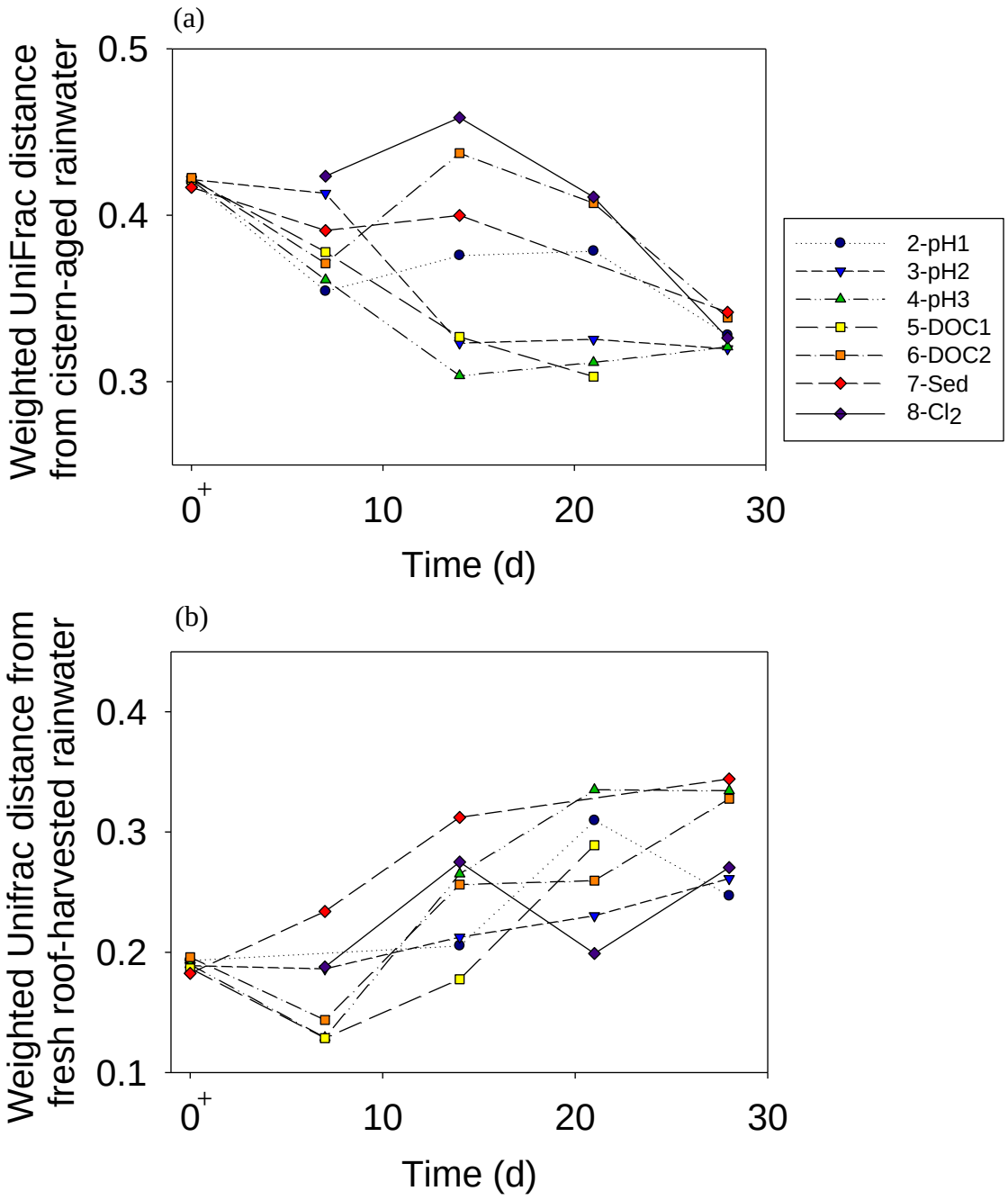

Figure 5.11: Weighted UniFrac distance of each sample from (a) cistern-aged rainwater, and from (b) fresh roof-harvested rainwater.

*Cistern 1-Cont is not shown because those samples are being resequenced.

*Samples with lower than 1075 sequences were not included in this plot (e.g., Cistern 8-Cl_2 at day 0^+).

Shared OTUs

As shown in Figure 5.12, the number of shared OTUs between each sample and fresh roof-harvested rainwater decreases over time, which is similar to the β-diversity analysis (Figure 5.11b).

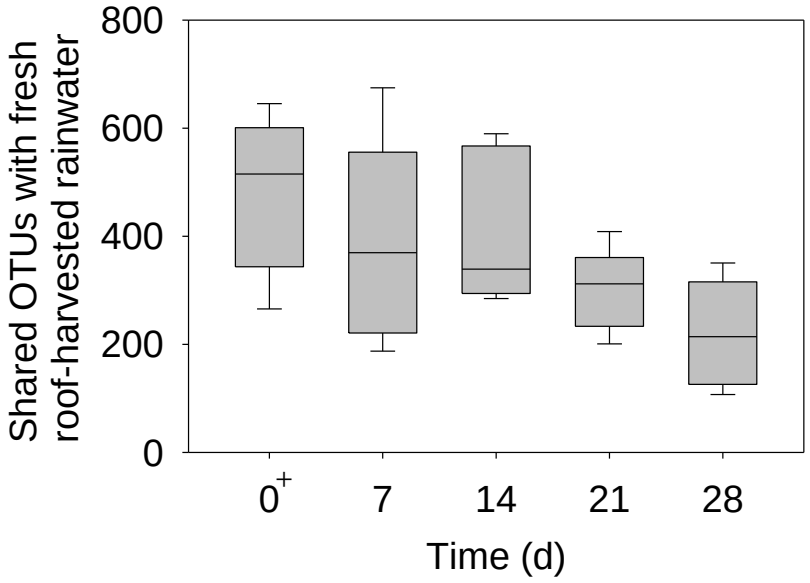

Figure 5.12: Shared operational taxonomic units (OTUs) between the bench-scale cisterns and fresh roof-harvested rainwater over time.

*Lines in boxes represent median values, vertical boxes represent 25th and 75th percentiles, error bars represent 10th and 90th percentiles.
*Samples with lower than 1075 sequences were not included in this plot (e.g., Cistern 8-Cl_2 at day 0^+).

Among all the samples

Principal Coordinate Analysis (PCoA) in 2-D was performed to visualize the weighted UniFrac distances for all the samples on one plot, and then the points from the same cistern were retrieved to create Figure 5.13 and Figure 5.14.

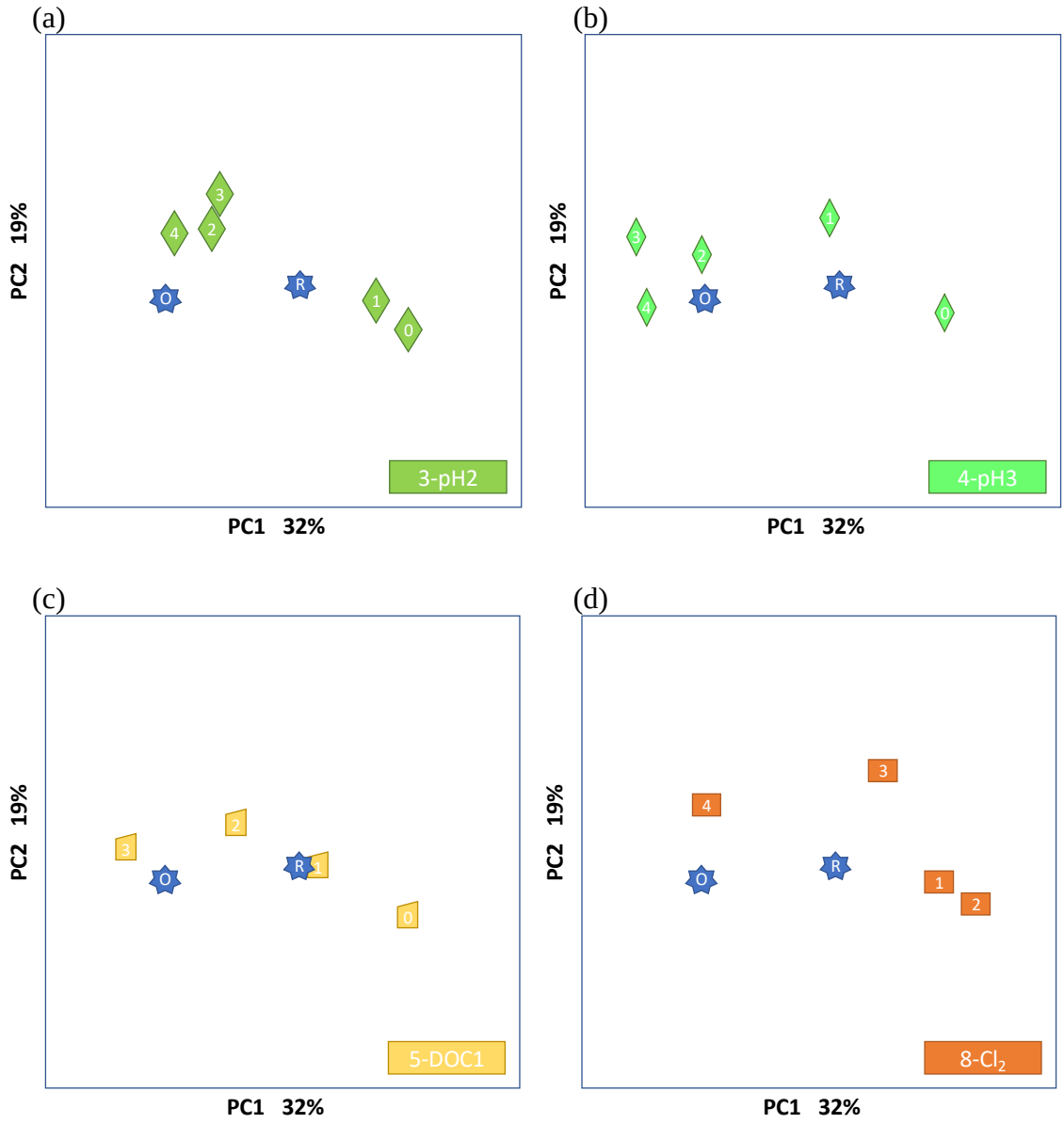

Figure 5.13: 2-D Principal Coordinate Analysis (PCoA) plot of weighted UniFrac distance for Cisterns (a) 3-pH2, (b) 4-pH3, (c) 5-DOC1, and (d) 8-Cl_2.

*Numbers 0, 1, 2, 3, and 4 represent days 0^+, 7, 14, 21, and 28, respectively. "O" represents cistern-aged rainwater, and "R" represents fresh roof-harvested rainwater.

*Samples with lower than 1075 sequences were not included in this plot (e.g., Cistern 8-Cl_2 at day 0^+).

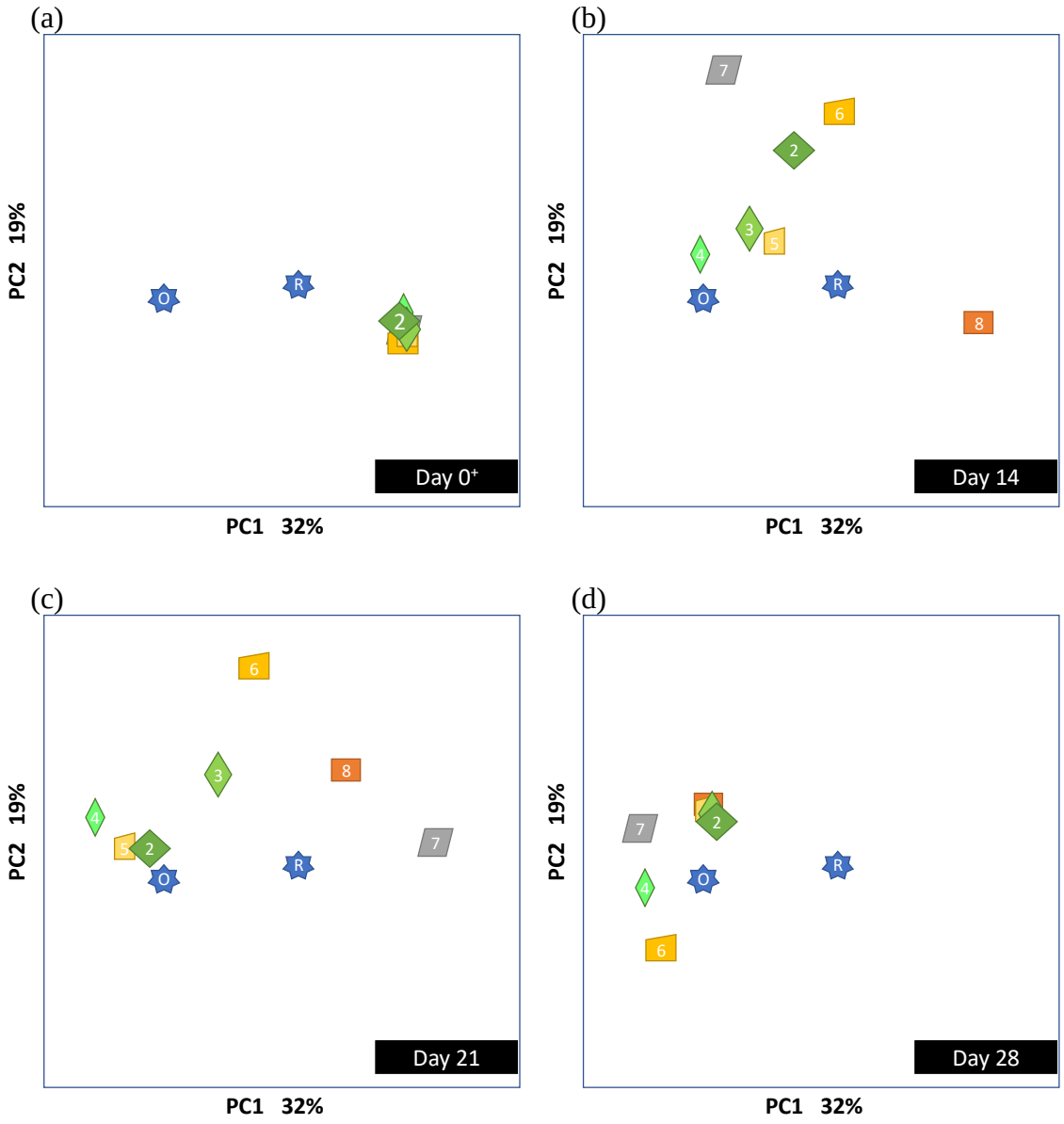

Figure 5.14: 2-D Principal Coordinate Analysis (PCoA) plot of weighted UniFrac distance on (a) day 0^{+}, (b) day 14, and (c) day 28.

*Numbers represent cistern numbers, "O" represents cistern-aged rainwater, and "R" represents fresh roof-harvested rainwater.

*Samples with lower than 1075 sequences were not included in this plot (e.g., Cistern 8-Cl_2 at day 0^{+}).

In this figure, each panel contains five cistern data points (marked as 0, 1, 2, 3, and 4 for days 0^+, 7, 14, 21, and 28, respectively), cistern-aged rainwater (marked as "O"), and fresh roof-harvested rainwater (marked as "R"). As also shown in Figure 5.11, Figure 5.13 showed that the communities shifted away from cistern-aged rainwater after the influx of fresh rain (moving closer to the community present in fresh rain), and then shifted back toward the community present in the cistern-aged rainwater over time. Cistern 8-Cl_2 showed slower recovery as compared to other cisterns (Figure 5.11, Figure 5.13).

Figure 5.14 shows data from all samples taken at the same point in time. As expected, all day 0^+ samples (panel a, which is right after the addition of fresh rainfall and *L. pneumophila*, and the physicochemical condition adjustment) had similar communities. However, over time, the communities diverge among the cisterns (panel b, which is after 14 days). At 28 days, the communities of many of the cisterns became similar to the cistern-aged rainwater (panel d).

5.3.5. Microbiome composition at the phylum level

As shown in Figure 5.15, Proteobacteria was a major phylum in the cistern-aged rainwater (85% as an average from all samples and sampling events), in the fresh roof-harvested rainwater (92%), and also in the ANSC sediment (77%). The full-scale RWH system samples in Task 2 (Chapter 4) also had Proteobacteria as a major phylum.

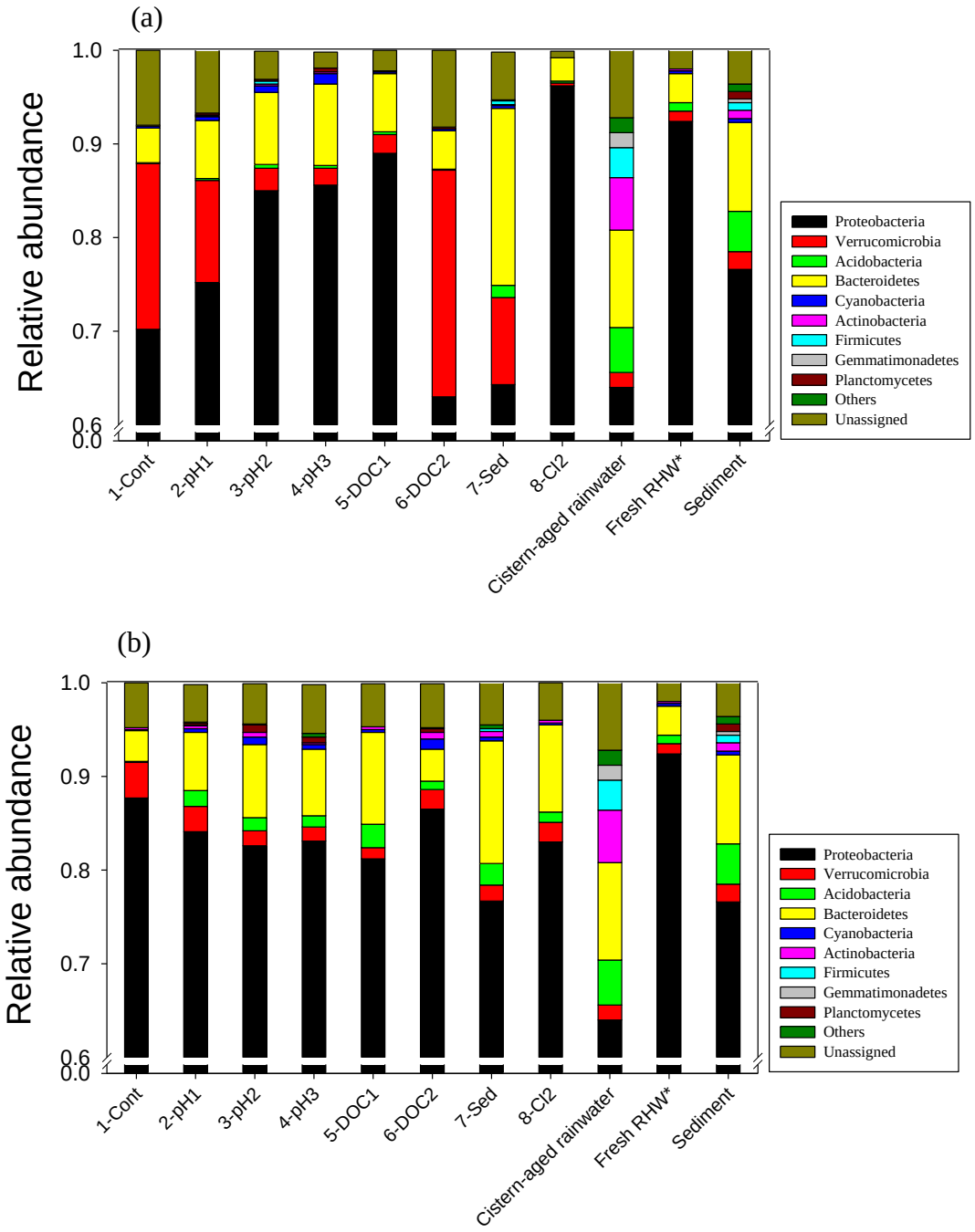

Figure 5.15: Phylum level microbiome composition on (a) day 14 and (b) day 28; the microbiomes of cistern-aged rainwater, fresh roof-harvested rainwater, and sediment also are shown for comparison.

*roof-harvested rainwater

The variance (σ^2) in abundance of each phylum (i.e., when the abundance of a specific phylum, such as Proteobacteria, was compared among all the cisterns at the same sampling event) became smaller from day 14 to day 28 (Table 5.5), which supports that the cistern microbiomes were becoming more similar to one another over time (Figure 5.14). At day 28, there was no noticeable difference in phylum-level composition based on the various physicochemical conditions in the cisterns (Figure 5.15).

Table 5.5: Variance (σ^2) in relative abundance of each phylum at days 0, 14, 21 and 28 among all the bench-scale cisterns

	Day 0	Day 14	Day 21	Day 28
Proteobacteria	27.49	148.26	77.61	11.22
Verrucomicrobia	0.01	75.58	56.15	0.69
Acidobacteria	0.02	0.15	0.19	0.60
Bacteroidetes	19.28	26.47	20.83	10.90
Cyanobacteria	0.00	0.12	0.04	0.11
Actinobacteria	0.12	0.01	0.02	0.04
Firmicutes	1.94	0.03	0.00	0.01
Gemmatimonadetes	0.01	0.00	0.01	0.00
Planctomycetes	0.01	0.02	0.10	0.10

Task 2 (Chapter 4) showed that the full-scale chlorinated cistern had different phylum-level composition as compared to the other cisterns (Figure 4.5a, where Planctomycetes or Cyanobacteria has higher relative abundance than does Proteobacteria). However, the chlorinated cistern that was monitored in Task 2 had been chlorinated recently relative to the sampling time, such that the cistern contained measurable chlorine residual (from 0.09 to 1.75 mg/L), while there was no chlorine residual in the current task

on and after day 7. Thus, in the current task, Cistern 8-Cl_2 had a similar microbiome to the other cisterns at day 28 (Figure 5.15b).

On average from all the samples, Bacteroidetes was the second most common phylum. However, on day 0^+ at Cistern 8-Cl_2, no Bacteroidetes were found (average in other cisterns: 3.5% of the microbiome), but instead more Firmicutes (4.0%) were found (average in other cisterns: 0.1%) as shown in Table 5.6. This pattern of lower abundance of Bacteroidetes and higher abundance of Firmicutes at the chlorinated cistern (when residual chlorine was present) as compared to non-disinfected cisterns also was found in the full-scale cisterns (Task 2, Chapter 4; Figure 4.5). However, this shift in composition was only temporary in the bench-scale cistern at day 0^+; over time, the relative abundance of Firmicutes and Bacteroidetes in Cistern 8-Cl_2 became similar to the other bench-scale cisterns (Table 5.6). Comparisons of relative abundance at the phylum-level are quite broad; therefore, in subsequent sections, family and genus-level studies were done to examine changes in the microbiome composition.

Table 5.6: Relative abundance (%) of Bacteroidetes and Firmicutes at Cistern 8-Cl_2 as compared to their average relative abundance in other cisterns

		Day 0^+	Day 7	Day 14	Day 21	Day 28
Cistern 8-Cl_2	Bacteroidetes	0.0	0.9	2.5	4.0	9.3
	Firmicutes	4.0	0.0	0.0	0.0	0.0
Other cistern median	Bacteroidetes	2.0	6.1	6.2	8.2	7.1
	Firmicutes	0.0	0.0	0.0	0.0	0.0

5.3.6. Microbiome composition at the family and genus level

If the relative abundances at the genus level from all the cisterns at the same sampling event were averaged, the relative abundance of *Sediminibacterium* spp. increased over time (Table 5.7). *Sediminibacterium* spp. also was the most common genus in the full-scale RWH cisterns on average (data not shown). *Sediminibacterium* spp. are commonly isolated from soil and eutrophic reservoir sediments (Qu & Yuan 2008), but they are not known to include human pathogens. The bench-scale data suggest that *Sediminibacterium* spp. can proliferate in stagnant cistern rainwater because their relative abundance increases while HPC (a surrogate for total bacterial numbers) were stable over 28 days. On average, the relative abundance of *Novosphingobium* spp. decreased over time (Table 5.7). From Task 2 (Chapter 4), a positive correlation between the relative abundance of *Novosphingobium* spp. and *L. pneumophila* in chlorinated rainwater was found (data not shown), and OTUs belonging to Sphingomonadaceae (a family that includes *Novosphingobium* spp.) also were positively rank-correlated with *L. pneumophila* in non-chlorinated rainwater (Table 4.4).

Table 5.7: Median relative abundance (%) of *Sediminibacterium* and *Novosphingobium* over time across all the cisterns

Genus	Cistern-aged rainwater	Fresh RWH*	Sediment	Day 0^{+}	Day 7	Day 14	Day 21	Day 28
Sediminibacterium	0.80	1.20	3.20	0.55	1.65	4.05	3.70	5.60
Novosphingobium	3.20	5.10	1.30	6.50	4.65	1.35	1.40	1.15

*roof-harvested rainwater

In the previous section (5.3.2), chlorination effectively controlled *L. pneumophila* in the cistern. Cistern 8-Cl_2 also was the cistern that had unique OTUs (sometimes

temporarily) as compared to the other cisterns. In this study, *Clostridium*, a genus that contains human pathogens, comprised 2.0% of the microbiome in Cistern 8-Cl_2 on day 0^+ but was not detected afterward. *Clostridium* was rarely detected in other cisterns (4 out of 35 samples at 0.1-0.2%). Chlorination also lowered the relative abundance of *Methylobacterium*, though the overall relative abundance of this genus was quite small in all cisterns; the average relative abundance of *Methylobacterium* in Cistern 8-Cl_2 (*Med* = 0.1%) was significantly lower than that in the other cisterns (*Med* = 0.2%), $Z = 1.68$, $p = 0.05$. Several species of *Methylobacterium* were reported to infrequently infect immunocompromised people and cause clinical manifestations such as fever (Truant et al. 1998).

5.3.7. Conclusion

In this chapter, the harvested rainwater microbiome was analyzed in eight bench-scale cisterns over a 28-d period. Each cistern was prepared with cistern-aged rainwater from a full-scale RWH system; then each system was spiked with fresh roof-harvested rainwater (20% of the final cistern volume) and *L. pneumophila*. The physicochemical condition (i.e., pH, DOC concentration, sediment presence, and chlorination) in each cistern was adjusted except for one control cistern. The following conclusions were drawn:

1. The water quality effect of any physicochemical condition adjustment (sodium carbonate for pH, organic tea for DOC concentration, sediment, chlorine) was temporary: pH, DOC concentration, turbidity and chlorine concentration recovered to their near-original levels within one week.
2. HPC were stable over 28 days in Cistern 1-Cont, the control cistern to which no changes in physicochemical conditions were made, meaning that rainwater

can support its own microbiome. DOC addition temporarily increased HPC, on day 4, and chlorination temporarily decreased HPC on day 0^{+}.

3. One-time chlorination effectively controlled *L. pneumophila* in the cistern with no regrowth through day 28. The *L. pneumophila* concentration decreased over time in all non-chlorinated cisterns. Higher pH cisterns seemed to facilitate slower decay of *L. pneumophila* through day 7 (when pH dropped from ca. 8.0 to 7.4). When the pH stayed near 7.4 after day 7, the *L. pneumophila* concentration dropped in those cisterns as well.
4. The microbiome of cistern-aged rainwater and fresh roof-harvested rainwater were distinct from one another. After an influx of fresh roof-harvested rainwater to each bench-scale cistern containing aged rainwater from a full-scale cistern, the microbiome of the cistern water became closer to that of fresh rain; over time, the microbiome became closer to cistern-aged rainwater (i.e., microbiome proceeded toward the pre-disturbance state). Relatedly, the number of shared OTUs between each cistern microbiome and fresh roof-harvested rainwater decreased over time.
5. Chlorination temporarily impacted the relative abundance of OTUs in the Firmicutes (e.g., *Clostridium* spp.). The chlorinated cistern proceeded toward its pre-disturbance microbiome more slowly than did other cisterns, but community richness was similar to other cisterns. Other than chlorination, condition adjustments (pH, DOC, sediment) did not induce noticeable differences in community richness and community recovery.

6. TASK CONCLUSIONS AND FUTURE WORK

6.1. TASK CONCLUSIONS

Rainwater harvesting (RWH) is an alternative method of providing water for indoor domestic use. Potential human pathogens have been found in harvested rainwater, but the microbiological quality of cistern and treated rainwater at residential sites has not been well-documented. In this dissertation, the following research questions were addressed:

1. How does residential-scale treatment change the physical, chemical, and microbiological quality of harvested rainwater?
2. Are indicator bacteria or heterotrophic plate counts (HPC) correlated with potential pathogens in harvested rainwater?
3. What physicochemical parameters are correlated with the microbiological quality of harvested rainwater?
4. What is the microbiome of harvested rainwater?
5. Does site/treatment/season influence the microbiome of harvested rainwater?
6. Does the cistern microbiome proceed towards its pre-disturbance state after an influx of fresh rainfall?
7. Does the pH, dissolved organic carbon (DOC) concentration, presence of sediment, or use of chlorination in the cistern impact community richness, community recovery, and *Legionella pneumophila* persistence after an influx of fresh rainfall to the cistern?

To address questions 1-5, rainwater that was harvested and treated at residential sites (by chlorination and filtration or filtration and ultraviolet [UV] disinfection) for indoor domestic use was surveyed for its physical and chemical quality and microbiome. Those

measurements included temperature, pH, turbidity, dissolved organic carbon (DOC), dissolved oxygen (DO), residual chlorine, total trihalomethanes (TTHM), heterotrophic plate counts (HPC), indicator bacteria (total coliform [TC], *Escherichia coli*, and enterococci), and potential human pathogens (*L. pneumophila*, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Aspergillus flavus*, *Aspergillus fumigatus*, and *Aspergillus niger*). To address questions 6 and 7, the harvested rainwater microbiome was analyzed in eight bench-scale cisterns over 28 days. Each cistern containing aged harvested rainwater was perturbed by the addition of fresh rainwater and *L. pneumophila*, and the physicochemical condition of each cistern was adjusted for pH, DOC concentration, sediment presence, or chlorine use.

Corresponding to questions above, the following conclusions were drawn:

1. Residential-scale treatment generally improved the physical, chemical, and microbiological quality of harvested rainwater. The RWH site practicing batch chlorination in the cistern generally had lower HPC at the finished-water tap as compared to that at the UV-disinfected sites. Even though *L. pneumophila* Sg 2-15 was not detected in the chlorinated cistern, it was detected downstream at the cold, finished-water tap; this suggests that the distribution system was colonized by *L. pneumophila*, which could act as a long-term reservoir of this potential human pathogen. At the UV sites, the unacceptably low decreases in bacterial concentrations from the rainwater cistern to the tap (after filtration, UV disinfection, and distribution) suggest that more conservative design, operation, and maintenance guidelines are needed for safe potable RWH.

2. No positive rank-correlations were observed between the non-fecal potential pathogens examined in this study (i.e., *L. pneumophila, M. avium*, *M. intracellulare*, *A. flavus*, *A. fumigatus*, and *A. niger*) and typical fecal indicator bacteria (i.e., TC, *E. coli*, and enterococci) at the full-scale RWH sites. Further, these potential pathogens were sometimes detected in treated rainwater samples via DNA-based methods even when no indicator bacteria were detected via most probable number (MPN) analyses. Thus, consistent with the rainwater literature, these data show that fecal indicator bacteria are insufficient to indicate non-fecal pathogens. By contrast, HPC and *L. pneumophila* concentrations were positively rank-correlated at non-chlorinated cisterns.

3. DOC concentration was positively rank-correlated with HPC, *L. pneumophila*, and *A. niger* concentrations in the non-chlorinated cisterns receiving water from Galvalume® roofs.

4. The microbiome of harvested rainwater is diverse. Proteobacteria, common in aquatic environments, was the most common phylum in non-chlorinated harvested rainwater cisterns. At genus-level classification, genera containing potential pathogens including *Mycobacterium*, *Legionella*, *Acinetobacter*, *Pseudomonas*, *Clostridium*, and *Staphylococcus* were detected from both treated and non-treated rainwater.

5. The most significant factor influencing the microbiome in full-scale RWH systems was the site, rather than the treatment or season. The cistern microbial communities

were distinct among the full-scale RWH sites, although they were located within 1 km of one another. At the residential RWH sites, the chlorinated cistern had a unique microbiome at each sampling event (which occurred after recent chlorination). However, the microbiomes of the non-disinfected cisterns were very stable over the period of a year, suggesting the resilience of the cistern microbiome to disturbances. The microbiome after filtration/UV-treatment/distribution was different from the microbiome in the associated cistern. Two genera containing potential human pathogens (*Staphylococcus* and *Clostridium*) were found more frequently in treated (UV-disinfected or chlorinated) rainwater samples as compared to untreated rainwater.

6. The cistern microbiome proceeded towards its pre-disturbance state after an influx of fresh roof-harvested rainwater. Even though the cistern-aged rainwater and fresh roof-harvested rainwater were collected from the same site, their microbiomes were distinct from one another. After an influx of fresh rainwater to each bench-scale cistern containing cistern-aged rainwater, the microbiome of the cistern water became closer to that of fresh roof-harvested rainwater; over time, the microbiome became closer to that of cistern-aged rainwater (proceeding toward the pre-disturbance state).

7. Among the four physicochemical condition adjustments (when adjusted only once), chlorination impacted microbiome the most. Chlorination effectively controlled *L. pneumophila* in the cistern until the end of the experiment (day 28) and temporarily impacted HPC and the relative abundance of operational taxonomic units (OTUs)

in the Firmicutes (e.g., *Clostridium* spp.). The *L. pneumophila* concentration decreased over time in all the cisterns. Other than chlorination, condition adjustments (pH, DOC concentration, sediment presence) did not induce noticeable differences in community richness and community recovery over a 28-d period.

6.2. FUTURE WORK

6.2.1. Better understand microbial risk

In this study, DNA of the harvested rainwater microbiome was analyzed with MiSeq®, and opportunistic pathogens were quantified by quantitative, real-time polymerase chain reaction (qPCR; targeting the 16S/18S rRNA gene). These methods are widely used in environmental studies, but there are several ways to improve their use: (1) distinguish between dead cells and live cells, (2) seek more than the phylogenetic distribution from DNA, and (3) quantitatively analyze microbial risk.

Dead cells and live cells

A common problem with DNA studies is that they do not usually discriminate between dead and live cells because DNA from dead cells can persist for weeks (Nocker et al. 2007). Future work should aim to better distinguish between dead and live microorganisms, especially when rainwater has been UV or chlorine-treated. To distinguish live cells from dead cells, several different existing methods can be used. First, longer amplicon can be adopted. In the current study, V4/V5 primers (Task 2) and V4 (Task 3) primers were used, which produced amplicon less than 400 base pairs (bp). Targeting longer amplicon might distinguish live and dead cells better especially when dead cells have DNA damage. Pyrosequencing (454) can generate longer amplicon (600

bp), but it has higher per-base error rate and is more costly (Nelson et al. 2014). Second, propidium monoazide (PMA), which can inhibit amplification of DNA from membrane-damaged cells (Bae & Wuertz 2009) during the polymerase chain reaction, can be used in conjunction with microbial community analyses to target live cells.

Beyond phylogenetic information

MiSeq® can provide an overall phylogenetic distribution for a microbial community. However, it does not provide phylogenetic information to the species level but only to the genus level at most. In the current study, less than half of OTUs found were identified to the genus level, and others were identified to the family or order levels. Moreover, phylogenetic distribution does not provide information about community function. Shotgun metagenomics is a new way to analyze both phylogeny and function at the same time (Sharpton 2014, Verberkmoes et al. 2009), which might aid our understanding of the rainwater microbiome.

Quantitative microbial risk assessment

Microbial risk associated with the use of roof-harvested rainwater at residential/commercial buildings should be studied. Detection of opportunistic pathogens (e.g., by qPCR) does not definitively address virulence, because different serogroups have different epidemic potentials. Even if some pathogens are transmitting disease, each person has a different dose-response relationship. To exploit qPCR results, the virulence of each pathogen and dose-response of different groups of people should be studied at the same time in a quantitative microbial risk assessment (ILSI 2000).

6.2.2. Futures studies regarding rainwater harvesting

Understanding* L. pneumophila *in rainwater cisterns

DOC concentration and HPC were positively rank-correlated with *L. pneumophila* concentrations. The potential for limiting the occurrence/concentration of *L. pneumophila* and other pathogens in harvested rainwater via removal of overhanging vegetation and regular gutter/cistern cleaning should be evaluated; such activities might directly impact the introduction of pathogens to the cistern or indirectly impact pathogen concentration by affecting bioavailable DOC input to the cistern. Regarding HPC, additional work to determine if there is a threshold HPC below which viable *L. pneumophila* do not occur in harvested rainwater should be performed. The relative abundance of some OTUs (e.g., an OTU belonging to Caulobacteraceae family) also had a positive correlation with the concentration of *L. pneumophila*. More studies might be needed to find microorganisms that support *L. pneumophila* proliferation in rainwater cisterns.

One of the motivations of Task 3 was to determine why similar RWH systems have different microbiomes. I had originally hypothesized that simple physicochemical condition adjustments in the cistern could cause major shifts in the microbiome. However, when the cistern physicochemical condition was adjusted once, the impact was not substantial enough to shift microbiome composition. However, this one-time physicochemical adjustment does not reflect the continuing periodic adjustments that are performed (for pH or chlorine) or occur (for DOC intrusion in each rainfall event) in real RWH systems. Therefore, continuous adjustments, rather than one-time adjustment, should be performed to test how physicochemical conditions affect microbiome in the real system.

APPENDIX

Accuracy

Accuracy of pH and turbidity measurement was tested before Task 1 by measuring standard reference s three times after the calibration. To check the accuracy of pH measurement, the pH meter was calibrated with three points, pH 4, 7, and 10, and each standard was measured three times again. To test accuracy of turbidity, a one-point calibration with the turbidity standard reference material of 0.61 NTU was used. Turbidity accuracy was calculated as follows:

$$Accuracy = \frac{average\ \ measurement\ \ of\ \ standard\ \ reference}{standard\ \ reference} \times 100\%$$

As shown in Table Apx 1, pH and turbidity accuracy were within the acceptable ranges.

Table Apx 1: Accuracy test result

	Read # 1	Read # 2	Read # 3	Average	Accuracy (pH units)	Target accuracy (pH units)
pH 4 standard	3.93	3.91	3.9	3.91	-0.09	± 0.1
pH 7 standard	6.98	6.97	6.97	6.97	-0.03	± 0.1
pH 10 standard	10.01	10.02	10.01	10.01	0.01	± 0.1

	Read # 1	Read # 2	Read # 3	Average	Accuracy (%)	Target accuracy (%)
Turbidity 0.61 NTU* standard	0.61	0.61	0.61	0.61	100	80-120
Turbidity 10 NTU standard	10	10	10	10	100	80-120

*NTU: nephelometric turbidity units

Limit of detection (LOD)

The limit of detection (LOD) for turbidity and chlorine measurements were established before Task 1 by using a blank fortified at a concentration two to three times the estimated instrument detection limit. Seven replicate aliquots of the fortified blank were processed through the entire analytical method. The LOD was calculated as follows:

$$\text{LOD} = \ t_{(\text{n}-1,1-\alpha=0.99)} \ \times \ S$$

Where:

S = standard deviation of the replicate analyses

n = number of replicates

$t_{(n-1,1-\alpha=0.99)}$ = student's t-value for a one-sided 99% confidence level and a standard deviation estimate with n-1 degrees of freedom; $t_{(6,0.99)} = 3.143$

The LOD for turbidity and chlorine concentration are shown in Table Apx 2.

Table Apx 2: Limit of detection (LOD) test result

	#1	#2	#3	#4	#5	#6	#7	Average	S[a]	LOD	Unit
Turbidity	0.26	0.24	0.26	0.26	0.29	0.27	0.27	0.26	0.015	0.048	NTU[b]
Chlorine	0.06	0.06	0.1	0.07	0.06	0.05	0.07	0.07	0.016	0.050	mg/L

[a]S: standard deviation of the replicate analyses

[b]NTU: nephelometric turbidity units

To determine an LOD for *Legionella pneumophila* qPCR analyses (Task 3), 6-point serial dilutions were made from 10^{-4} to 10^{-7} ng/µL, and each dilution was measured in seven replicate in 30-µL reaction. The lowest concentration where obtained at least four threshold cycles (C_t) with lower standard deviation of C_t than 0.3 was chosen as LOD.

Most probable number (MPN) quality control

Standards were directly purchased from IDEXX (Westbrook, ME; part number 98-29000-00 for Colilert®, 98-29002-00 for Enterolert®). As shown in Table Apx 3, Colilert® did not react to non-coliform, and the fluorescent probe for *E. coli* did not react to non-*E. coli* coliform such as *Klebsiella pneumoniae*. Enterolert® did not react to non- enterococci. In terms of enumeration, test readings were all lower than the test standard concentration, but they are all in the acceptable range specified by the manufacturer.

Table Apx 3: Most probable number (MPN) quality control result for Colilert® and Enterolert®

Colilert®	Standard concentration	Acceptable range (MPN/100 mL)	Measured total coliform concentration (MPN/100 mL)	Measured *E. coli* concentration (MPN/100 mL)
E. coli	130	36 – 224	37.2	37.2
Klebsiella pneumoniae	64	25 – 103 (only for total coliform)	31.8	0
Pseudomonas aeruginosa	0	0	0	0

Enterolert®	Standard concentration	Acceptable range (MPN/100 mL)	Measured enterococci concentration (MPN/100 mL
Enterococcus faecalis	102	43 – 161	75.4
E. coli	0	0	0
Streptococcus bovis	0	0	0

Precision

The precision of pH and temperature measurements were reported as the absolute range (D) of duplicate measurements:

$$D = |first\ \ measurement - second\ \ measurement|$$

The precision of duplicate measurements was calculated with relative percent difference (RPD), and the precision of triplicate measurements was calculated with relative standard deviation (RSD) as follow:

$$RPD\ \ (\%) = \frac{difference\ \ between\ \ two\ \ measurements}{average\ \ of\ \ two\ \ measurements} \times 100$$

$$RSD\ \ (\%) = \frac{standard\ \ deviation\ \ (S)}{average\ \ of\ \ three\ \ measurements} \times 100$$

Very few samples were out of compliance for precision during the whole study (data not shown). Table Apx 4 shows average precision data from the first quarterly sampling at Task 1 and qPCR precision at Task 3. All other tests showed similar precision.

Table Apx 4: Average precision during the first quarterly sampling at Task 1 (temperature to heterotrophic plate count) and at Task 3 (qPCR)

	Average	Unit	Target
Temperature	0.0		±0.1
Turbidity	7.5	(%)	25%
pH	0.2		±0.5
Dissolved oxygen	0.4	(%)	25%
Chlorine	2.5	(%)	25%
Heterotrophic plate count	56.3	(%)	200%
qPCR (*Legionella pneumophila*) threshold cycle	1.1	(%)	-

GLOSSARY

ANSC	Austin Nature and Science Center
ARCSA	American Rainwater Catchment Systems Association
ATP	adenosine triphosphate
BCYE	Buffered Charcoal Yeast Extract
CFU	colony-forming unit
DOC	dissolved organic carbon
DO	dissolved oxygen
FC	fecal coliform
fg	femtogram
h	hour(s)
HPC	heterotrophic plate count
km	kilometer(s)
LOD	limit of detection
LOQ	limit of quantification
L	liter(s)
μL	microliter(s)
MCL	maximum contaminant level
Mdn	median
mg	milligram(s)
mJ/cm^2	millijoule(s) per square centimeter
mL	milliliter(s)
mm	millimeter(s)
MPN	most probable number

NTU	nephelometric turbidity units
NTM	nontuberculous mycobacteria
OTUs	operational taxonomic units
PCoA	principal coordinate analysis
QIIME	Quantitative Insight into Microbial Ecology
r_s	spearman's rho
qPCR	quantitative, real-time polymerase chain reaction
RWH	rainwater harvesting
Sg	serogroup
SA:V	surface area to volume ratio
TC	total coliform
TTHM	total trihalomethanes
UV	ultraviolet
USEPA	United States Environmental Protection Agency

REFERENCES

Abbasi, T. & Abbasi, S.A., 2011. Sources of pollution in rooftop rainwater harvesting systems and their control. *Critical Reviews in Environmental Science and Technology*, 41:23:2097.

Abdel-Nour, M., Duncan, C., Low, D.E. & Guyard, C., 2013. Biofilms: the stronghold of *Legionella pneumophila*. *International Journal of Molecular Sciences*, 14:11:21660.

Ahmed, W., Brandes, H., Gyawali, P., Sidhu, J.P.S. & Toze, S., 2014. Opportunistic pathogens in roof-captured rainwater samples, determined using quantitative PCR. *Water Research*, 53:361.

Ahmed, W., Gardner, T. & Toze, S., 2011. Microbiological quality of roof-harvested rainwater and health risks: a review. *Journal of Environment Quality*, 40:1:13.

Ahmed, W., Goonetilleke, A. & Gardner, T., 2010a. Implications of faecal indicator bacteria for the microbiological assessment of roof-harvested rainwater quality in Southeast Queensland, Australia. *Canadian Journal of Microbiology*, 56:6:471.

Ahmed, W., Hodgers, L., Sidhu, J.P.S. & Toze, S., 2012. Fecal indicators and zoonotic pathogens in household drinking water taps fed from rainwater tanks in Southeast Queensland, Australia. *Applied and Environmental Microbiology*, 78:1:219.

Ahmed, W., Huygens, F., Goonetilleke, A. & Gardner, T., 2008. Real-time PCR detection of pathogenic microorganisms in roof-harvested rainwater in Southeast Queensland, Australia. *Applied and Environmental Microbiology*, 74:17:5490.

Ahmed, W., Vieritz, A., Goonetilleke, A. & Gardner, T., 2010b. Health risk from the use of roof-harvested rainwater in Southeast Queensland, Australia, as potable or nonpotable water, determined using quantitative microbial risk assessment. *Applied and Environmental Microbiology*, 76:22:7382.

Albrechtsen, H., 2002. Microbiological investigations of rainwater and graywater collected for toilet flushing. *Water Science & Technology*, 46:6-7:311.

American Public Health Association (APHA), A.W.W.A.A., and Water Environment Federation (WEF), 2004. Standard methods for the examination of water and wastewater (9215C). 21st ed. American Public Health Association.

Amin, M. & Han, M., 2009. Roof-harvested rainwater for potable purposes: application of solar collector disinfection (SOCO-DIS). *Water Research*, 43:20:5225.

Bae, S. & Wuertz, S., 2009. Discrimination of viable and dead fecal *Bacteroidales* bacteria by quantitative PCR with propidium monoazide. *Applied and Environmental Microbiology*, 75:9:2940.

Berjeaud, J.-M., Chevalier, S., Schlusselhuber, M., Portier, E., Loiseau, C., Aucher, W., Lesouhaitier, O. & Verdon, J., 2016. *Legionella pneumophila*: The Paradox of a Highly

Sensitive Opportunistic Waterborne Pathogen Able to Persist in the Environment. *Frontiers in Microbiology*, 7:486.

Beumer, A., King, D., Donohue, M., Mistry, J., Covert, T. & Pfaller, S., 2010. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in drinking water and biofilms by quantitative PCR. *Applied and Environmental Microbiology*, 76:21:7367.

Bodey, G.P. & Vartivarian, S., 1989. Aspergillosis. *European Journal of Clinical Microbiology and Infectious Diseases*, 8:5:413.

Brewer, M., 2011. United States drought monitor. <http://droughtmonitor.unl.edu/mapsanddata/maparchive.aspx>.

Bull, A.T., 2011. Actinobacteria of the extremobiosphere. *Extremophiles Handbook*:1203.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K. & Gordon, J.I., 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7:5:335.

Carter, J.T., Rice, E.W., Buchberger, S.G. & Lee, Y., 2000. Relationships between levels of heterotrophic bacteria and water quality parameters in a drinking water distribution system. *Water Research*, 34:5:1495.

Chang, M., McBroom, M.W. & Beasley, R.S., 2004. Roofing as a source of nonpoint water pollution. *Journal of Environmental Management*, 73:4:307.

Chern, E.C., King, D., Haugland, R. & Pfaller, S., 2015. Evaluation of quantitative polymerase chain reaction assays targeting *Mycobacterium avium*, *M. intracellulare*, and *M. avium* subspecies paratuberculosis in drinking water biofilms. *Journal of Water and Health*, 13:1:131.

Chidamba, L. & Korsten, L., 2015. Pyrosequencing analysis of roof-harvested rainwater and river water used for domestic purposes in Luthengele village in the Eastern Cape Province of South Africa. *Environmental Monitoring and Assessment*, 187:2:1.

Cho, B.C. & Jang, G.I., 2014. Active and diverse rainwater bacteria collected at an inland site in spring and summer 2011. *Atmospheric Environment*, 94:0:409.

Covert, T.C., Rodgers, M.R., Reyes, A.L. & Stelma, G.N., 1999. Occurrence of nontuberculous mycobacteria in environmental samples. *Applied and Environmental Microbiology*, 65:6:2492.

DeSantis, T., Hugenholtz, P., Keller, K., Brodie, E., Larsen, N., Piceno, Y., Phan, R. & Andersen, G.L., 2006. NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Research*, 34:suppl 2:W394.

Dillaha III, T.A. & Zolan, W.J., 1985. Rainwater catchment water quality in Micronesia. *Water Research*, 19:6:741.

Donohue, M.J., O'Connell, K., Vesper, S.J., Mistry, J.H., King, D., Kostich, M. & Pfaller, S., 2014. Widespread molecular detection of *Legionella pneumophila* Serogroup 1 in cold water taps across the United States. *Environmental Science & Technology*, 48:6:3145.

East, R., 2013. Microbiome: soil science comes to life. *Nature*, 501:7468:S18.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C. & Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*, 27:16:2194.

Evans, C.A., Coombes, P.J., Dunstan, R.H. & Harrison, T., 2009. Extensive bacterial diversity indicates the potential operation of a dynamic micro-ecology within domestic rainwater storage systems. *Science of the Total Environment*, 407:19:5206.

Falkinham, J.O., 1996. Epidemiology of infection by nontuberculous mycobacteria. *Clinical Microbiology Reviews*, 9:2:177.

Falkinham, J.O., Norton, C.D. & LeChevallier, M.W., 2001. Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other mycobacteria in drinking water distribution systems. *Applied and Environmental Microbiology*, 67:3:1225.

Farreny, R., Morales-Pinzón, T., Guisasola, A., Taya, C., Rieradevall, J. & Gabarrell, X., 2011. Roof selection for rainwater harvesting: quantity and quality assessments in Spain. *Water Research*, 45:10:3245.

Ferroni, G.D. & Kaminski, J.S., 1980. Psychrophiles, psychrotrophs, and mesophiles in an environment which experiences seasonal temperature fluctuations. *Canadian Journal of Microbiology*, 26:10:1184.

Festing, H., Gallet, D., Liu, H., Shorofsky, B., Becker, S., Kilpatrick, R. & Nichols, K., 2013. The case for fixing the leaks: protecting people and saving water while supporting economic growth in the Great Lakes region.

Fierer, N. & Jackson, R.B., 2006. The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences of the United States of America*, 103:3:626.

Ghisi, E. & Ferreira, D.F., 2007. Potential for potable water savings by using rainwater and greywater in a multi-storey residential building in southern Brazil. *Building and Environment*, 42:7:2512.

Gilbert, J.A., Steele, J.A., Caporaso, J.G., Steinbrück, L., Reeder, J., Temperton, B., Huse, S., McHardy, A.C., Knight, R. & Joint, I., 2012. Defining seasonal marine microbial community dynamics. *The ISME journal*, 6:2:298.

Haugland, R.A., Varma, M., Wymer, L.J. & Vesper, S.J., 2004. Quantitative PCR analysis of selected *Aspergillus*, *Penicillium* and *Paecilomyces* species. *Systematic and Applied Microbiology*, 27:2:198.

Hayes, S.L., Sivaganesan, M., White, K.M. & Pfaller, S.L., 2008. Assessing the effectiveness of low-pressure ultraviolet light for inactivating *Mycobacterium avium* complex (MAC) micro-organisms. *Letters in Applied Microbiology*, 47:5:386.

He, X., Tian, Y., Guo, L., Lux, R., Zusman, D.R. & Shi, W., 2010. Oral-derived bacterial flora defends its domain by recognizing and killing intruders—a molecular analysis using Escherichia coli as a model intestinal bacterium. *Microbial Ecology*, 60:3:655.

Helmreich, B. & Horn, H., 2009. Opportunities in rainwater harvesting. *Desalination*, 248:1-3:118.

Hicks, L.A., Garrison, L.E., Nelson, G.E. & Hampton, L.M., 2011. Legionellosis – United States, 2000-2009. Morbidity and mortality weekly report.

Hijnen, W.A.M., Beerendonk, E.F. & Medema, G.J., 2006. Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo) cysts in water: a review. *Water Research*, 40:1:3.

Hilborn, E.D., Covert, T.C., Yakrus, M.A., Harris, S.I., Donnelly, S.F., Rice, E.W., Toney, S., Bailey, S.A. & Stelma, G.N., 2006. Persistence of nontuberculous mycobacteria in a drinking water system after addition of filtration treatment. *Applied and Environmental Microbiology*, 72:9:5864.

Hunicke-Smith, S.P., 2015. rRNA bacterial gene and fungal ITS metagenomics samples. <https://wikis.utexas.edu/display/GSAF/rRNA+bacterial+gene+and+fungal+ITS+metagenomics+samples>, June 16.

ILSI, R., 2000. Revised framework for microbial risk assessment.

Ji, P., Parks, J., Edwards, M.A. & Pruden, A., 2015. Impact of water chemistry, pipe material and stagnation on the building plumbing microbiome. *PloS One*, 10:10:e0141087.

Johnson, D.R., Lee, T.K., Park, J., Fenner, K. & Helbling, D.E., 2014. The functional and taxonomic richness of wastewater treatment plant microbial communities are associated with each other and with ambient nitrogen and carbon availability. *Environmental Microbiology*.

Jung, D., 2015. What is happening at the roof top?! <http://m.ohmynews.com/NWS_Web/Mobile/Ten/report_last.aspx?atcd=A0002150594&srscd=0000011376>.

Karim, M.R., 2010. Microbial contamination and associated health burden of rainwater harvesting in Bangladesh. *Water Science and Technology*, 61:8:2129.

Kaushik, R., Balasubramanian, R. & Dunstan, H., 2014. Microbial quality and phylogenetic diversity of fresh rainwater and tropical freshwater reservoir. *PloS One*, 9:6:e100737.

Keithley, S.E., 2012. The effect of treatment on the quality of harvested rainwater M.Sc. .

Kim, J.S., Lee, K.C., Kim, D.S., Ko, S.H., Jung, M.Y., Rhee, S.K. & Lee, J.S., 2015. Pyrosequencing analysis of a bacterial community associated with lava-formed soil from the Gotjawal forest in Jeju, Korea. *Microbiology Open*, 4:2:301.

Kim, M. & Han, M., 2011. Composition and distribution of bacteria in an operating rainwater harvesting tank. *Water Science and Technology*, 63:7:1524.

Kim, T.-S., Jeong, J.-Y., Wells, G.F. & Park, H.-D., 2013. General and rare bacterial taxa demonstrating different temporal dynamic patterns in an activated sludge bioreactor. *Applied Microbiology and Biotechnology*, 97:4:1755.

Kobayashi, M., Oana, K. & Kawakami, Y., 2014. Incidence of *Legionella* and heterotrophic bacteria in household rainwater tanks in Azumino, Nagano prefecture, Japan. *Microbiology and Immunology*, 58:1:15.

Koide, M., Higa, F., Tateyama, M., Cash, H.L., Hokama, A. & Fujita, J., 2014. Role of Brevundimonas vesicularis in supporting the growth of Legionella in nutrient-poor environments. *The New Microbiologica*, 37:1:33.

Kool, J.L., Carpenter, J.C. & Fields, B.S., 1999. Effect of monochloramine disinfection of municipal drinking water on risk of nosocomial Legionnaires' disease. *The Lancet*, 353:9149:272.

Korajkic, A., Parfrey, L.W., McMinn, B.R., Baeza, Y.V., VanTeuren, W., Knight, R. & Shanks, O.C., 2015. Changes in bacterial and eukaryotic communities during sewage decomposition in Mississippi river water. *Water Research*, 69:0:30.

Kuczynski, J., Costello, E.K., Nemergut, D.R., Zaneveld, J., Lauber, C.L., Knights, D., Koren, O., Fierer, N., Kelley, S.T. & Ley, R.E., 2010. Direct sequencing of the human microbiome readily reveals community differences. *Genome Biology*, 11:5:210.

Kueneman, J.G., Parfrey, L.W., Woodhams, D.C., Archer, H.M., Knight, R. & McKenzie, V.J., 2014. The amphibian skin-associated microbiome across species, space and life history stages. *Molecular Ecology*, 23:6:1238.

Långmark, J., Storey, M.V., Ashbolt, N.J. & Stenström, T.-A., 2005. Accumulation and fate of microorganisms and microspheres in biofilms formed in a pilot-scale water distribution system. *Applied and Environmental Microbiology*, 71:2:706.

Lee, J.Y., Yang, J.-S., Han, M. & Choi, J., 2010. Comparison of the microbiological and chemical characterization of harvested rainwater and reservoir water as alternative water resources. *Science of the Total Environment*, 408:4:896.

Li, Z., Boyle, F. & Reynolds, A., 2010. Rainwater harvesting and greywater treatment systems for domestic application in Ireland. *Desalination*, 260:1:1.

Lian, T., Jin, J., Wang, G., Tang, C., Yu, Z., Li, Y., Liu, J., Zhang, S. & Liu, X., 2017. The fate of soybean residue-carbon links to changes of bacterial community composition in Mollisols differing in soil organic carbon. *Soil Biology and Biochemistry*, 109:50.

Lin, Y., Li, D., Zeng, S. & He, M., 2016. Changes of microbial composition during wastewater reclamation and distribution systems revealed by high-throughput sequencing analyses. *Frontiers of Environmental Science & Engineering*, 10:3:539.

Lozupone, C. & Knight, R., 2005. UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*, 71:12:8228.

Lye, D.J., 1987. Bacterial levels in cistern water systems of northern Kentucky. *Water Resources Bulletin*, 23:6:1063.

Mendez, C.B., Klenzendorf, J.B., Afshar, B.R., Simmons, M.T., Barrett, M.E., Kinney, K.A. & Kirisits, M.J., 2011. The effect of roofing material on the quality of harvested rainwater. *Water Research*, 45:5:2049.

Meulemans, C.C.E., 1987. The basic principles of UV–disinfection of water. *Ozone: Science & Engineering*, 9:4.

Moissl-Eichinger, C., Auerbach, A.K., Probst, A.J., Mahnert, A., Tom, L., Piceno, Y., Andersen, G.L., Venkateswaran, K., Rettberg, P. & Barczyk, S., 2015. Quo vadis? Microbial profiling revealed strong effects of cleanroom maintenance and routes of contamination in indoor environments. *Scientific Reports*, 5.

Navas-Molina, J.A., Peralta-Sánchez, J.M., González, A., McMurdie, P.J., Vázquez-Baeza, Y., Xu, Z., Ursell, L.K., Lauber, C., Zhou, H. & Song, S.J., 2013. Advancing our understanding of the human microbiome using QIIME. *Methods in Enzymology*, 531:371.

Nelson, M.C., Morrison, H.G., Benjamino, J., Grim, S.L. & Graf, J., 2014. Analysis, optimization and verification of Illumina-generated 16S rRNA gene amplicon surveys. *PloS One*, 9:4:e94249.

Nocker, A., Sossa-Fernandez, P., Burr, M.D. & Camper, A.K., 2007. Use of propidium monoazide for live/dead distinction in microbial ecology. *Applied and Environmental Microbiology*, 73:16:5111.

Nolasco, J., 2011. Sustainable water management for urban agriculture: planting justice, Oakland.

Nourmoradi, H., Nikaeen, M., Stensvold, C.R. & Mirhendi, H., 2012. Ultraviolet irradiation: an effective inactivation method of Aspergillus spp. in water for the control of waterborne nosocomial aspergillosis. *Water Research*, 46:18:5935.

Novitsky, J.A., 1986. Degradation of dead microbial biomass in a marine sediment. *Applied and Environmental Microbiology*, 52:3:504.

Park, S.-J., Bae, S.-Y., Jin, J.-H., Suh, J.-h. & Park, S.-C., 2006. Ubiquitous water recycle management service proposal in ubiquitous city. *Artificial Reality and Telexistence Workshops*:558.

Pepper, I.L., Rusin, P., Quintanar, D.R., Haney, C., Josephson, K.L. & Gerba, C.P., 2004. Tracking the concentration of heterotrophic plate count bacteria from the source to the consumer's tap. *International Journal of Food Microbiology*, 92:3:289.

Pinto, A.J., Schroeder, J., Lunn, M., Sloan, W. & Raskin, L., 2014. Spatial-temporal survey and occupancy-abundance modeling to predict bacterial community dynamics in the drinking water microbiome. *MBio*, 5:3:e01135.

Pinto, A.J., Xi, C. & Raskin, L., 2012. Bacterial community structure in the drinking water microbiome is governed by filtration processes. *Environmental Science & Technology*, 46:16:8851.

Qu, J.-H. & Yuan, H.-L., 2008. *Sediminibacterium salmoneum* gen. nov., sp. nov., a member of the phylum *Bacteroidetes* isolated from sediment of a eutrophic reservoir. *International Journal of Systematic and Evolutionary Microbiology*, 58:9:2191.

Salas, J.C., Cortesi, L., König, K.W., Malmer, A., Prasad, E. & Sharma , B., 2009. Rainwater harvesting: a lifeline for human well-being. 978-92-807-3019-7, Division of Environmental Policy Implementation, United Nations Environment Programme (UNEP).

Sazakli, E., Alexopoulos, A. & Leotsinidis, M., 2007. Rainwater harvesting, quality assessment and utilization in Kefalonia Island, Greece. *Water Research*, 41:9:2039.

Scalf, A., 2013. Tornado-stricken south Kenton gets $1M grant. *cincinnati.com*.

Schets, F., Italiaander, R., Van Den Berg, H. & de Roda Husman, A., 2010. Rainwater harvesting: Quality assessment and utilization in The Netherlands. *Journal of Water and Health*, 8:2:224.

Sharpton, T.J., 2014. An introduction to the analysis of shotgun metagenomic data. *Frontiers in Plant Science*, 5.

Simmons, G., Hope, V., Lewis, G., Whitmore, J. & Gao, W., 2001. Contamination of potable roof-collected rainwater in Auckland, New Zealand. *Water Research*, 35:6:1518.

Simmons, G., Jury, S., Thornley, C., Harte, D., Mohiuddin, J. & Taylor, M., 2008. A Legionnaires' disease outbreak: A water blaster and roof-collected rainwater systems. *Water Research*, 42:6:1449.

Smit, E., Leeflang, P., Gommans, S., van den Broek, J., van Mil, S. & Wernars, K., 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. *Applied and Environmental Microbiology*, 67:5:2284.

States, S.J., Conley, L.F., Kuchta, J.M., Oleck, B.M., Lipovich, M.J., Wolford, R.S., Wadowsky, R.M., et al, 1987. Survival and multiplication of *Legionella pneumophila* in municipal drinking water systems. *Applied and Environmental Microbiology*, 53:5:979.

Steffen, J., Jensen, M., Pomeroy, C.A. & Burian, S.J., 2013. Water supply and stormwater management benefits of residential rainwater harvesting in US cities. *Journal of the American Water Resources Association*, 49:4:810.

Stout, J.E., Yu, V.L. & Best, M.G., 1985. Ecology of *Legionella pneumophila* within water distribution systems. *Applied and Environmental Microbiology*, 49:1:221.

Streit, W.R. & Schmitz, R.A., 2004. Metagenomics – the key to the uncultured microbes. *Current Opinion in Microbiology*, 7:5:492.

Thomas, J.M. & Ashbolt, N.J., 2010. Do free-living amoebae in treated drinking water systems present an emerging health risk? *Environmental Science & Technology*, 45:3:860.

Thomas, R.B., Kirisits, M.J., Lye, D.J. & Kinney, K.A., 2014. Rainwater harvesting in the United States: a survey of common system practices. *Journal of Cleaner Production*, 75:166.

Thomas, V., Herrera-Rimann, K., Blanc, D.S. & Greub, G., 2006. Biodiversity of amoebae and amoeba-resisting bacteria in a hospital water network. *Applied and Environmental Microbiology*, 72:4:2428.

Truant, A.L., Gulati, R., Giger, O., Satishchandran, V. & Caya, J.G., 1998. Methylobacterium species: an increasingly important opportunistic pathogen. *Laboratory Medicine*, 29:11:704.

Tuffley, R.E. & Holbeche, J.D., 1980. Isolation of the *Mycobacterium avium-M. intracellulare-M. scrofulaceum* complex from tank water in Queensland, Australia. *Applied and Environmental Microbiology*, 39:1:48.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A. & Affourtit, J.P., 2009. A core gut microbiome in obese and lean twins. *Nature*, 457:7228:480.

TWDB, 2012. 2012 Water for Texas:Texas Water Development Board

USEPA, 1995. Method 524.2. Measurement of purgeable organic compounds in water by capillary column gas chromatography/mass spectrometry. Revision 4.1.

Verberkmoes, N.C., Russell, A.L., Shah, M., Godzik, A., Rosenquist, M., Halfvarson, J., Lefsrud, M.G., Apajalahti, J., Tysk, C. & Hettich, R.L., 2009. Shotgun metaproteomics of the human distal gut microbiota. *The ISME journal*, 3:2:179.

Vesper, S.J., Haugland, R., Rogers, M. & Neely, A., 2007. Opportunistic *Aspergillus* pathogens measured in home and hospital tap water by quantitative PCR (QPCR). *Journal of Water and Health*, 5:3:427.

von Reyn, C.F., Marlow, J.N., Arbeit, R.D., Barber, T.W. & Falkinham III, J.O., 1994. Persistent colonisation of potable water as a source of *Mycobacterium avium* infection in AIDS. *The Lancet*, 343:8906:1137.

Wadowsky, R.M., Wolford, R., McNamara, A. & Yee, R.B., 1985. Effect of temperature, pH, and oxygen level on the multiplication of naturally occurring *Legionella pneumophila* in potable water. *Applied and Environmental Microbiology*, 49:5:1197.

Wang, Q., Garrity, G.M., Tiedje, J.M. & Cole, J.R., 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, 73:16:5261.

Wang, S., Dong, H., Hou, W., Jiang, H., Huang, Q., Briggs, B.R. & Huang, L., 2014. Greater temporal changes of sediment microbial community than its waterborne counterpart in Tengchong hot springs, Yunnan Province, China. *Scientific Reports*, 4.

Wang, Y. & Qian, P.-Y., 2009. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PloS One*, 4:10:e7401.

Winchell, J.M. & Benitez, A.J., 2013. Methods and compositions for detection of legionella:PCT/US2014/038441.

Yudelson, J., 2010. *Dry run- preventing the next urban water crisis (Chapter 9. Brown water)*.

Zwart, G., Crump, B.C., Kamst-van Agterveld, M.P., Hagen, F. & Han, S.-K., 2002. Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquatic Microbial Ecology*, 28.